

nature*September 18, 1975*

No easy way to solve universities' problems

IN 1970 the number of students entering universities in the United Kingdom to study dentistry (1,170) was a mere third of the number planning to study chemistry. The 1974 entry was somewhat different, according to the statistical supplement to the Twelfth Report of the Universities Central Council on Admissions (UCCA). There were 3,065 prospective dentists, one and a half times the number of prospective chemists. Provisional figures for 1975 show the trend halted, but no more.

This is the most extreme example. The swing would be somewhat less if, say, medical students and physicists were compared, but the point must surely now have penetrated the consciousness of even the most optimistic or ostrich-like scientists: that a totally unplanned manpower shortage of scientists is creeping up on us.

Ironically, at the same time an equally unplanned shortage of job opportunities within universities has arisen, with the unwillingness of most universities to take on long term staff commitments during the past two years. It would be incorrect to see these two shortfalls balancing each other out and so preventing the worst from happening; only a small fraction of university entrants eventually opt for a university career. The universities are in trouble, and university science is in the most trouble of all.

It is against this depressing backdrop that the House of Commons Select Committee on Science and Technology issues its interim report on the short-term problems of scientific research in British Universities (House of Commons Paper 504, HMSO, 65p). Nothing in the report will be a great surprise; the committee has not fallen for any one witness nor has it advanced any patent remedy of its own. Instead there is a sober assessment of the situation and some muted pleas to the Department of Education and Science and the University Grants Committee to hold the line on the balance between teaching and research in universities and to the universities to look carefully at their individual priorities.

Research in universities looks a precariously marginal activity when viewed from Whitehall. The Department of Education and Science is very dominantly a department of education. The University Grants Committee sees research and educational elements as indistinguishable when it allocates money, but reckons research only to comprise "rather more than 25%" of the totals. The research councils put less than one-third of their money into university departments by way of grants, studentships and fellowships. In every way university research is vulnerable to the stirrings of its larger administrative

bedfellows. Some of those bedfellows, such as academic salaries, not only rightly take precedence, but also may be committed for the next 10, 20 or more years.

And yet a crisis need not be a bad thing, particularly in an environment which has known nothing but growth for years. The committee itself remarks that if inflation concentrates university minds, it may be of some benefit. The opportunity to review priorities and seek the most efficient means of deploying more limited funds is one which it would be "highly regrettable" for the universities to ignore. Linked to this broad hint is a similar one that universities must review the relevance of their research activities. This statement is a bit of a compromise; there was at one time within the committee a feeling that there should be a national body—perhaps a 'de-grants committee'—to perform this function. And it is made clear that this idea is only temporarily on ice.

Can and should the universities respond to such a challenge to assess their priorities both in general and in terms of relevance? The answer is almost certainly that individually they lack the tools to do so or indeed much perception of how to assess relevance. The vice-like grip of job security so severely restricts the universities' freedom of action that opportunities for change, redeployment or tapering off in the staffing of departments is restricted, even in good times, largely to the arbitrariness of death, resignation or retirement. Certain economies are no doubt still possible by sharing of facilities and so on, but when 80% or more of the universities' general funds are committed to salaries and wages it is clear that the scope is limited if personnel levels are maintained.

Even more difficult to respond to is the challenge that universities must review relevance. First, there would certainly be sniping across the great divide between arts and sciences; is mediocre electrical engineering less or more relevant than competent Old Testament theology? Second, some of the relevancies can only be seen in a national perspective; maybe mathematical geodesy or Amharic look pretty irrelevant in a local context, but maybe there is a national need for a few graduates a year. Third, demands for relevance could lead to the sight of academics presenting unedifying inflated and misleading claims for their work rather in the way that confessions are 'volunteered' in some countries.

The committee rightly identifies the principal crisis as one of confidence in the future. A veiled threat to review relevance before the committee does it for you will hardly bolster this confidence. □



THE POOR CLIMBING BOYS.

A HUMANE British Parliament passed an Act several years ago, known as Lord Shaftesbury's "CLIMBING BOYS' ACT," whereby the barbarous employment of poor children for sweeping chimneys was prohibited, and the use of machines enforced. So far as London is concerned, this Act is, we believe, faithfully enforced,—but in the provinces there are at the present time upwards of 3000 of these poor creatures still suffering the wrongs of British slavery.

As the bulk of the climbing boys come from the families of working-men, who by accident, or the premature death of their parents, are thrown into Union Houses—we appeal to our readers to do what they can to enforce the faithful carrying out of the Act of Parliament. Mr. Wm. Wood, of Bowden, Manchester, or Mr. James Glass, of 24, Barrington Crescent, Brix-



"PITY THE POOR CLIMBING BOYS!"

ton, will gladly correspond with any parties who desire to aid in preventing the use of climbing boys in districts where they are at present employed.

Cancer at work

Although there has been progress in the treatment of cancer by radiation, surgery, chemotherapy and immunotherapy, it is probable that efforts to prevent the disease have been more rewarding and effective than those made to improve treatment. Some occupational cancers can be prevented when the cause is identified. It is, however, often difficult to find the causes and sometimes even more difficult to remove them when they are known. Two hundred years after Percival Pott made the connection between cancer and the working environment, Professor E. Boyland reviews subsequent work on occupational cancers.

PERCIVAL POTT, FRS, was a surgeon at St Bartholomew's Hospital; his "Chirurgical observations relative to the cataract, the polypus of the nose, the cancer of the scrotum, the different kinds of ruptures and the modification of the toes and feet" was published by Hawes and Collins in Pater-noster Row in 1775. The paper entitled "cancer scroti" describes "a disease as peculiar to a certain set of people which has not, at least to my knowledge, been publicly noticed; I mean the chimney-sweepers' cancer". "The trade call it soot-wart. The disease, in these people, seems to derive its origin from the lodgement of soot in the rugae of the scrotum". He had identified a cause of cancer—a carcinogen active in man. The observations and identification did not stop the exposure of the child chimney sweeps, and Charles Kingsley, who was an active member of the Christian Socialist movement led by Frederick Denison Maurice, described the plights of these child workers in *Waterbabies* almost a century later.

Pott pointed out that "the subjects are young, in general in good health, at least at first, the disease brought on them by their occupation—all this makes it (at first) a very different case from cancer which appears in an elderly man, whose fluids become acrimonious from time, as well as other causes; or from the same kind of complaint in women who have ceased to menstruate. But be all this as it may, the scrotum is no vital organ, nor can the loss of a part of it ever be attended with . . . the smallest degree of inconvenience". He must have perceived the process which is chemical carcinogenesis.

Percival Pott was a modest scholar; as his son-in-law, Sir James Earle, wrote "he often said he began to teach when he had much to learn; and that he was not actuated by that opinionative wisdom which sometimes attends advanced life, after all his study and experience he confessed that he still retained a long list of inquerenda". Samuel Johnson was one of his patients and his portrait painted by Joshua Reynolds hangs in St Bartholomew's Hospital. His epitaph reads "The labours of the ancients were familiar to him; he scorned to teach a science of which he had not traced the growth."

The Romans had chimneys in connection with their hot air heating systems, but in northern Europe chimneys were developed slowly. In mediaeval times hearths in the centre of the living room and vents in the roof were usual. When coal became available as a domestic fuel Count Rumford established the forms and proper relationships of the parts of the chimney. The

chimneys which required children to clean them were an English development and the associated disease was more common in England than in continental Europe. It seems probable that chimney sweeps in Germany had some form of protective clothing.

Before Percival Pott, Ramazzini in the *de morbis artificum*, published in 1700, considered occupational diseases such as the colic due to lead to which painters, glaziers and plumbers were exposed. Ramazzini had noticed the high incidence of breast cancer in nuns and indicated that this was caused by their occupation.

Following the work of Pott many clinicians reported on the condition. Bell in 1794 described other cases and said it appears obviously to be produced by soot, for it is found that besides chimney sweeps those who are employed in manufacture in which soot enters are occasionally seized by it. James Earle in 1808 recorded a case of a gardener with epithelioma of the left hand on which he had previously hung a pot containing soot used to kill slugs. He saw that skin cancer elsewhere than in the scrotum could be caused by soot. In the edition of the works of Percival Pott, revised by James Earle in 1808, reference was made to an eight-year-old apprentice with scrotal cancer. Often, however, there was a long latent period. Curling in 1856 described a case of a sailor who developed scrotal cancer in the fifth decade of life but who had been brought up as a sweep. In the nineteenth century the disease remained but it was recognised that not all children employed as sweeps developed scrotal cancer.

With the expansion of the Industrial Revolution increasing amounts of oils were used for the lubrication of machines. Before 1850 the oils were mainly of animal origin but later mineral oils were used in much larger quantities. Volkmann in 1875 described occupational skin cancer by tar, paraffin and soot in Germany and in the following year (1876) Bell of Edinburgh described cases of "paraffin cancer" in workers in the Scottish shale oil industry.

As the incidence of chimney sweeps' cancer decreased in the nineteenth century the incidences of scrotal cancer increased among the spinners of the Lancashire cotton industry, the first cases being described in 1887. This "mule spinners' cancer" has decreased in the present century because (1) the cotton industry employs fewer workers, (2) ring spinning machines have replaced the old mule spinners, (3) less carcinogenic oils controlled by specifications are used, and (4) there has been a general improvement in hygiene. Although less common, scrotal cancer

still occurs in industry in conditions in which men are exposed to lubricating oils.

Although the first synthetic dyestuffs were made in England by Perkin, the German chemical industry was the first to exploit the discoveries and manufacture dyestuffs on a large scale. It was in Germany that the first cases of bladder cancer due to exposure to aromatic amines were reported by Rehn in 1895. The development of dyestuffs industries in other countries was associated with widespread increases in the incidence of bladder cancer. Cases of bladder cancer in men who had manufactured dyestuffs intermediates including 2-naphthylamine and benzidine in England were reported by Wignall in 1929. Another aromatic amine, 4-aminobiphenyl, had been manufactured and used in the United States for many years. The intelligent anticipation and laboratory work of Williams and his colleagues (1952), showing that it was carcinogenic to animals, prevented the manufacture of this compound in Britain and other countries. In 1955 evidence was presented showing that this compound had caused cancer in men occupied with its manufacture.

In addition to their use in dyestuff manufacture, aromatic amines were used in the rubber industry. Case and Hosker in 1954 found an increased



Pott by Reynolds (courtesy Bart's Hospital)

incidence of bladder cancer in rubber workers in one region of England. It is probable that carcinogenic aromatic amines have not been used in the rubber industry since 1950 and because of the possible hazard special facilities for early diagnosis have been available for 20 years. Cancer of the bladder has been a prescribed disease in Britain since 1953.

Early in the present century Japanese workers demonstrated that the application of coal tar to the ears of rabbits or the skin of mice induced tumours some of which were malignant. This provides a bioassay of carcinogenic activity which Kennaway and his colleagues used to identify the hydrocarbon 1, 2,5, 6-dibenzanthracene as the first known compound to induce cancer and then for the isolation and characterisation of 3, 4-benzopyrene from coal tar. Carcinogenic aromatic amines and polycyclic hydrocarbons were known 40 years ago, but in spite of much research the mechanisms by which they exert their carcinogenic actions are still not exactly known.

The knowledge of the chemical nature of the carcinogens, which must have been present in the soot that caused cancer in the young chimney sweeps described by Percival Pott, has in some cases helped with the problems of reducing the hazard of environmental carcinogens. Many other extrinsic cancer-producing agents have been discovered. As most cancers in man are probably caused by extrinsic factors, many still remain to be identified. The discovery of these and the removal or reduction of them in the environment will decrease the incidence of cancer in the way started by the English surgeon two hundred years ago. □

THE young scientist who wants to venture into medical research now faces an acute problem in finding a satisfactory career structure. It used to be that workers in this field had some form of tenure, either through their contract or through a moral obligation readily acknowledged by the employer. But the trend in recent years has been to offer only temporary appointments lasting, at most, five years. This poses severe problems.

The really outstanding person will always find help elsewhere if the support runs out, of course. My concern is more for the capable and well qualified scientist. Three to five years of medical research may actually prove to be a handicap when competing for teaching or research posts with candidates who have pursued a more academic path.

The Medical Research Council have recently recognised the problem in their own establishments by ensuring that tenure will be granted to staff no later than in their mid-30s—other institutions will probably fall into line. But this only accounts for 20–30 people annually. Numerous first-rate scientists better equipped for work in applied medical research are still un-

helped by this scheme.

In the UK, experience has shown that the best way to effect the transition from research activity to clinical trials is through a team of medical and scientific staff who can meet

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regularly on an equal footing. (Contrast this with the United States where young doctors may undergo a long training in basic science and later become competent to direct highly advanced work in a hospital laboratory.) Such teams at present exist in advanced institutes and in them the scientist (who often makes the early running) has learnt to communicate successfully with the clinician. Might not such teams be introduced more widely in medical schools and general hospitals? They could be a highly efficient means of speeding the acceptance by clinicians of the most recent advances in laboratory methods. And might not this be a solution to the career problem for medical scientists?

Such a scheme could best start in specialised centres or medical schools where there already is a tradition of regular clinical conferences. Young medical staff would rapidly recognise the advantages of team work with scientists; it is all but impossible to keep pace with advanced laboratory methods as well as advanced medicine.

Recruitment of scientists to such a service could take place in two ways. Firstly, at the graduate level, for which the Universities might set up an M.Sc. course in Bio-Medical Science as a recognised qualification. Secondly, scientists who have already spent three to five years in medical research might be required to take a diploma in Bio-Medical Science. These courses would provide a general clinical background to the problems in which the hospital laboratories play a major role a large proportion of the lectures being given by medical staff. This would be far less expensive for the country than the training of medical graduates in advanced laboratory methods, in addition to their medical training. Such a scheme could provide a career structure for those scientists at present in medical research who have a most uncertain future.—E. J. Ambrose.

international news

As Britain prepares to face another winter with further rises in the price of fuel, the Department of Energy, formed less than two years ago to deal with the problems of energy crisis, has become the target of heavy criticism in a report prepared by the House of Commons Select Committee on Science and Technology (House of Commons Paper No. 487, HMSO, 1975). Among the proposals contained in the report is a call for new administrative machinery at Ministerial level to co-ordinate a policy intended to cut Britain's annual energy bill by £1,000 million (about 15%).

According to the chairman of the all-party committee, Mr Arthur Palmer, a reorganisation is needed because of the failure of the present energy administration to implement policies of energy conservation effectively. He said last week that very little had been done during the past year to improve energy efficiency and that the committee did not share the government view that the recent diminution in energy demand, largely attributable to the recession, represented a genuine saving. The problem of energy conservation, he said, had not been considered with sufficient urgency by the existing administration, apparently because of the tendency in government circles both to treat issues requiring practical and administrative attention in an abstract and political manner, and to consider important long term issues only in the light of their short term political implications.

In all, the report lists 42 recommendations for increasing efficiency in the use and supply of energy. Underlying the proposals is a recommendation that a full time "task force" of Ministers, officials and outside experts be set up to meet the problems of the continuing energy crisis. If the government decides to act on the committee's proposals after the report has been considered by Parliament, the new administration would replace the Advisory Council on Energy Conservation (ACEC) and the Advisory Council on Research and Development (ACORD) both of which are responsible to the Department of Energy at present. Nonetheless, that would not mean the demise of the Department of Energy itself, which would remain responsible for the more specific problems of coal, offshore oil and gas and nuclear power. Indeed, the report notes

British energy savers taken to task

by Allan Piper

that it may be the department's involvement with those matters that has led to what is seen as a lack of central direction and decisiveness in matters of conservation.

If it set up, the main function of the task force will be to ensure a greater efficiency of energy use in every area of the economy. Operating on its own budget, it will work within a time limit towards clearly defined objectives. On the basis of the information collected from expert witnesses interviewed during the preparation of the report, the committee suggests that a realistic target is a reduction in energy consumption of 15% within three years. That could be achieved, according to the report, "without sacrificing output, employment, or living standards". Additionally, the report further points out that an effectively directed programme of energy conservation could be sustained at a lower cost than the development of an equivalent amount of additional energy from North Sea resources.

The effectiveness of the task force will, it is hoped, rest mainly in its regional structure. Recognising the need for a more direct implementation of policies than there has been in the past, the committee believes that future policies can only be successful if the task force establishes a strong local organisation.

Exactly how the task force will fit in with the Department of Energy is not entirely clear, however, though the committee recommends that it should be set up at Ministerial level and should report directly to the Prime Minister. Questioned on the purpose of establishing another administrative body in addition to the Department of Energy, Mr Palmer said the committee felt it was necessary because the problems of energy conservation cut across existing departmental boundaries. As an example, he cited the Department of Industry and the Ministry of Defence.

Both, he said, are in need of effective policies of energy conservation, but neither falls within the jurisdiction of the Department of Energy. The new administration, on the other hand, would possess sufficient political status to implement effective policies wherever necessary.

On the industrial front the report calls for the introduction of a new grants scheme that should be particularly favourable to smaller interests and is designed to encourage the introduction of more efficient plant. Industry accounts for about 40% of British energy consumption, and the wastage is particularly high. It therefore seems likely that the committee's call for legislation to cover the industrial use of fuels and the running of industrial equipment will receive serious consideration. Furthermore, the benefits to be gained by using the experience of independent consultants on energy-saving schemes is recognised in the report, and the committee suggests that they could be suitably employed as agents by the task force. In the committee's view the National Industrial Fuel Efficiency Service (NIFES), the largest consultant organisation in the field, with 136 engineers, would be particularly well equipped to cope and could, even, be reintroduced advantageously to the public sector.

The committee has listed several recommendations for preventing domestic energy wastage and profligate use of energy in the areas of public and private transport. The report suggests that new buildings should be constructed to a specific standard of insulation, with legislation introduced to ensure the implementation of minimum requirements within the home. There is also a call for the maintenance of advertising campaigns to impress on the public the need for a greater awareness of the continuing energy crisis. In that respect the Department of Energy is criticised for its past record and is accused of following a policy "feeble in contrast to the need for strong action". One recommendation that seems long overdue is that the energy supply industries—the electricity and gas boards—must reconsider the scheme whereby consumers pay proportionally less for additional consumption beyond a certain point.

On the question of transport the committee makes the interesting suggestion that inland waterways and

As an example of how not to regulate environmental chemicals, the case of diethylstilboestrol (DES) is a tough one to beat. A feed additive which stimulates growth in cattle, DES has been enmeshed in bitter controversy for more than a decade. The problem is that DES is highly carcinogenic, and traces of it can sometimes be found in meat and beef liver. Consequently, after bending over backwards for years in an effort to find a way to keep DES on the market the Food and Drug Administration (FDA) in 1973 issued an order banning its use. But the ban was thrown out in court because the FDA had ineptly failed to follow the correct procedure when it passed sentence. The result is that DES is still on the market and is now found in beef liver at higher concentrations than when it was banned two years ago.

That sorry series of events prompted the Senate last week to pass an unprecedented bill banning the use of DES in cattle feed, at least until the FDA can prove that it is safe. If the House of Representatives follows suit—prospects there are uncertain—it will be the first time that Congress has legislated against a single chemical.

The case against DES is a complicated one, which involves weighing the potential risks of long term exposure to extremely low levels of a known carcinogen against the economic benefits of bringing cattle to market more quickly. It is not something at which politicians are particularly adept, and the debate certainly proved that the floor of the Senate is not a particularly good place to reach complex regulatory decisions.

Diethylstilboestrol is one of the few chemicals for which there is good evidence that it causes cancer in man. Recently there have been a number of cases—220 have come to light so far—of an extremely rare vaginal cancer in young women in their late teens and early 20s; a common factor among the women is that their mothers took DES as a drug during the late stages of pregnancy to prevent miscarriage (a practice now discontinued). With such evidence at hand, there is good reason to keep DES out of foods.

But DES is an extraordinarily good growth stimulant for cattle, and it originally seemed that if it was withdrawn from cattle feed a few days before the beasts were slaughtered, it would all be excreted and no residues would contaminate the meat. Things did not work out like that, however, because in spite of FDA regulations

Washington seen

by Colin Norman

requiring cattle to be taken off DES at least a week before slaughter, residues continued to show up in beef and liver.

The levels at which it is present are, however, extremely low, and here the discussion gets into the controversial area of whether or not there is a threshold dose of a carcinogen below which it presents no health hazard. The Senate debate proceeded with those supporting a ban, led by Senator Edward M. Kennedy, citing a sheaf of reports suggesting that known carcinogens should be kept out of the environment completely, while opponents of the ban argued that DES is present in meat at such lower levels that it poses no risk. In the end, the ban was approved by a vote of 61 to 29, and everybody was left wishing that the FDA had been less inept when it first tried to ban DES.

● Acting with a degree of unanimity that is rare in the top echelons of the scientific community, 186 eminent scientists have endorsed a statement condemning astrology as pernicious, anti-scientific nonsense based on magic and superstition. Published in the September/October issue of *The Humanist*, the journal of the American Humanist Association, the statement notes that astrology "pervades modern society" and suggests that "the time has come to challenge directly, and forcefully, the pretentious claims of astrological charlatans".

Proceeding from the observation that "in ancient times people believed in the predictions and advice of astro-

logers because astrology was part and parcel of their magical world view... [and] they had no concept of the vast distances from the Earth to the planets and stars", the statement notes that "now these distances can and have been calculated, we can see how infinitesimally small are the gravitational and other effects produced by the distant planets and the far more distant stars". Consequently, "It is simply a mistake to imagine that the forces exerted by stars and planets at the moment of birth can in any way shape our futures. Neither is it true that the position of distant heavenly bodies can make certain days or periods more favourable to particular kinds of action, or that the sign under which one was born determines one's compatibility or incompatibility with other people".

The statement was drafted by Bart J. Bok, Emeritus Professor of Astronomy at the University of Arizona, Lawrence Jerome, a science writer, and Paul Kurtz, Professor of Philosophy at the State University of New York at Buffalo. It was sent to about 300 scientists, chiefly astronomers and astrophysicists, during the summer, and about 60% of them responded positively. Among the signatories are 18 Nobel Prizewinners.

Given the eminently sensible and unarguable contents of the statement, it is not surprising that it attracted so much influential support. But why was it deemed necessary to open a frontal assault on astrology at this point in time? Kurtz said last week that he is disturbed by the burgeoning interest in astrology, particularly in the United States (where, according to one estimate, there are some 20,000 practising astrologers), and Bok noted in a separate article in *The Humanist* that some universities and junior colleges even offer courses in astrology. The statement itself also condemned "the continued uncritical dissemination of astrological charts, forecasts and horoscopes by the media and by otherwise reputable newspapers, magazines and book publishers [which] can only contribute to the growth of irrationalism and obscurantism".

coastal shipping routes should receive more extensive use than at present. Perhaps alarming to the private motorist will be the call for consideration of severe restrictions on the use of private transport in one or two selected cities—Mr Palmer mentioned Bristol, his home constituency as a suitable example. Mr Palmer also mentioned the committee's suggestion that companies should establish car pools rather than provide individuals with cars.

As for the less immediate future, the government is urged to consider the use of alternative sources of energy such as nuclear fusion and hydrogen fuel. Significant to that proposal is the recommendation that the Energy Technology Support Unit (ETSU), at Harwell, primarily responsible for research into new energy projects under the leadership of the Department of Energy's Chief Scientist, Dr Walter Marshall, should be expanded and

strengthened by the task force. Mr Palmer also mentioned what he referred to as "way out" sources, and said that in particular the committee had been interested by the idea of tidal power. The Severn Estuary is in fact well suited to provide energy from that source and has already been the subject of several research schemes. Presumably, solar energy and wave power will also come under scrutiny if the committee's proposals are adopted. □

THE right of the government of the host country to an international conference to refuse entry visas to foreign delegates has long been a vexed one. The Tbilisi conference on artificial intelligence (September 2-8) produced an interesting variation on this problem: the right of the government of the host country to refuse to admit its own nationals.

The problem in this case was that of Jewish refusenik scientists, still living within the Soviet Union but dismissed from their academic posts while awaiting a visa for Israel. Although the Soviet scientific hosts of the conference were quite willing to admit them, the KGB was totally opposed to this and considered their wish to attend as being a "provocation". The international organising committee, however, made strong representations that, should these scientists be refused permission to attend, there would be forceful protests and resulting adverse publicity. Consequently, for fear of a withdrawal by the USA and other Western delegations, and as a result of last minute meetings in Moscow, the authorities decided to permit Dr Aleksandr Lerner to travel from Moscow to attend the conference. He was escorted to Tbilisi by the same KGB officer who had formally told him he could not go and welcomed by the head of the Cybernetics Institute of the Georgian Academy of Sciences, Dr V. V. Chavchanidze, who had himself photographed pinning on Lerner's conference badge. Although subjected to a certain amount of official surveillance throughout the conference, Lerner was able to circulate freely among the delegates and take part in

a panel session "Cybernetics, Mathematics and Artificial Intelligence".

Less fortunate were the Gold'dshtein brothers, Isai and Grigori. Being residents of Tbilisi, they did not have the initial difficulties of travel permits and hotel registrations; nevertheless, after meeting with newly arrived delegates and talking with them on scientific

Soviet diary

from Vera Rich

topics and also on the difficulties of life as a refusenik, they were called in by the KGB and warned to stay away from the area of the conference building, unless they had had an official invitation from the conference committee. It was not until Saturday, September 6, the penultimate working day of the conference, that Elizaveta Bykova, the wife of Isai and herself a physicist, managed to convey this message to the committee. After a long and forceful meeting between the conference committee and the Georgian hosts, a compromise was reached by which Dr Chavchanidze undertook to invite them for the remainder of the conference on his own personal initiative—a singular example of moral courage.

This reluctance to admit the refuseniks seems to be related to the attitude to Israeli scientists. No scientists resident in Israel were allowed to attend, and one Israeli, Dr Y. Yakimovsky, at present working in the USA, received a promise of a visa far too late for him to attend. Nevertheless, two other

Israelis from the USA, Judah Pearl and Meir Weinstein, were permitted to attend, so that the Soviet authorities fulfilled at least the letter of the unofficial working agreement on international conferences—that although individual delegates may be refused entry, no national delegation will be excluded *in toto*.

● The Presidium of the Supreme Soviet of the USSR has announced that "The Order of Friendship of Nations" has been awarded, individually, to each of the Academies of Science of the Union Republics of the USSR, namely, to the Ukrainian, Byelorussian, Uzbek, Kazakh, Georgian, Azeri, Lithuanian, Moldavian, Latvian, Kirgiz, Tadjik, Armenian, Turkmenian, and Estonian Academies, "for service in the development of Soviet science, economics, and culture, and in the training of highly qualified scientific cadres". One Republic only is not so honoured—the Russian Federated Republic, which has no academy of its own.

● Reconstruction work has begun at the highest botanical garden in the world, at Khorog in Tadjikistan. The garden, at a site in the High Pamirs some 2000 m above sea-level ("above the clouds") is extensively used for field work on the introduction and acclimatisation of plants in extremal conditions. The plants, received on a basis of a regular exchange of seed with 30 Soviet and 36 foreign botanical gardens, therefore include not only and the Hindu Kush, but also more high-altitude flora from the Himalayas plebeian species, including several strains of potatoes, and that mainstay of agricultural planning of the Khrushchev era—maize.

THERE are indications that the new head of the Soviet space establishment, Academician Roald Sagdeyev, has wider interests in space cooperation than costly "spectaculars". Space scientists of the West first made his acquaintance at this summer's COSPAR Symposium in Bulgaria (COSPAR is shorthand for the ICSU Committee for Space Research, the principal international scientific—as opposed to technological—meeting ground). The impression he made was fresh and favourable: he is youngish, full of ideas, and expresses them in excellent English. This is not quite the image projected by his long-surviving predecessor, Academician Blagdonravov—a loveable octogenarian if ever there was one—who died earlier this year.

Sagdeyev's actual post as head of the USSR Institute of Space Research in Moscow puts him in a key position to monitor international developments at

Plans for space

from Angela Croome

least in the physical sciences. Soviet development of ideas for experiments must surely have seemed to stagnate since the 1950s; sputniks were becoming not only relatively smaller but stupider. At the COSPAR meeting, Sagdeyev was not only responsive to the idea of international cooperation in certain fields but showed particular interest in getting involved in X-ray astronomy projects.

On the organisational front, the initiative seems to have passed from the USA (at least temporarily). The formation of the European Space Agency (ESA) as a matching body to NASA has thrown into prominence the lack of a learned body in Europe, comparable with the Space Sciences Board of the US National Academy of Sciences, to give informed but inde-

pendent opinions on the long term objectives of space research. A European Space Sciences Board has now been formed under the formal auspices of the European Science Foundation with Sir Harrie Massey as chairman.

It acts as a clearing house for research ideas in conjunction with the US Academy of Sciences' Board and looks ahead to acting as a joint negotiating body for the West for broad cooperative programmes with the Soviet Union on a range of topics better pursued on a synoptic or systematic basis than from discrete and occasional national spacecraft. The idea of an international space observatory seems particularly to recommend itself to Sagdeyev and his institute colleagues at present. The European Space Sciences Board holds its next meeting—the third—in London in the middle of September, and expects to clarify some of these possibilities. □

correspondence

The WHO and mosquitoes

SIR,—We have read with interest your editorial (July 31) on the allegations made by a section of the Indian press and the Public Accounts Committee of the Indian Parliament against the Delhi-based Research Unit on Genetic Control of Mosquitoes whose activities were jointly implemented and supervised by the World Health Organisation and the Indian Council of Medical Research (ICMR).

With regard to the technical aspects of this controversy, you have highlighted the crucial fact that the authors of the allegations failed to read the abundant literature issued by the research unit, either reports or publications, which were made freely available to them, and that they used logic so tenuous that it does not stand up to unprejudiced examination. We might add that they did not even take the trouble to discuss the controversial points with the many Indian scientists carrying out research at the unit.

We should like, however, to complement your analysis by supplying some additional information on other aspects of the controversy, for which the only documentation that you had was that presented by the authors of the allegations.

Dr Jayaraman's assertion that he was refused information on the project by the WHO because "it was sensitive to the Indian press" does not represent the truth. On the contrary, all co-operation was extended to him and a meeting was immediately arranged under the chairmanship of the Director-General of the ICMR with the Director of the National Institute of Communicable Diseases of India, the unit's Project Leader and Dr Pal of the WHO's Vector Biology and Control Unit at Headquarters. The Chairman invited Dr Jayaraman to raise any questions about the unit's research after he had a chance to see the special issue of the *Journal of Communicable Diseases* devoted to papers on the unit's work. It was most unfortunate that Dr Jayaraman never availed himself of this offer; had he done so, the fallacies in his subsequent published statements might have been avoided. You have also quoted Dr Jayaraman's allegation that he was "indirectly sounded out for a job as an Information Officer at WHO Headquarters", the implication being that this was done in an effort to appease him. No such offer was made

to him; what was offered was the full cooperation of the WHO information services in the preparation of his article. It is noteworthy that Dr Jayaraman did not make this allegation in the press, but only before the Parliamentary Committee.

With reference to the use of chemosterilised mosquitoes, your presentation of the situation is not entirely accurate. The unit's statement that thiotepa residues break down very rapidly in the bodies of mosquitos is not a mere "claim" but is based on investigations, the results of which have been published (LaBrecque, G. C., Bowman, M. C., Patterson, R. S., and Seawright, J. A., *Bull. Wld Hlth Org.*, 47, 675-676; 1972). The unit's statement that drinking-water wells were never used for the release of chemosterilised pupae is factually correct. Apart from considerations of safety, the use of drinking-water wells (in which *Culex fatigans* does not normally breed) would have defeated the aim of releasing sterile males at the natural breeding sites, which in the area and season concerned are disused irrigation wells. The toxic effects of the well water on laboratory-reared pupae was discovered not because these were released in the wells, but because treated pupae were placed for emergence in this water in containers which in the initial stages were floating and later suspended above the water.

You sharply criticise WHO's handling of public relations during the controversy. We may point out that as an international organisation the WHO does not make any statement which could be construed as intervention in an internal dispute or a matter falling within the domestic jurisdiction of a member state. So long as this was the case, and the question was under investigation by a Parliamentary Committee in India, it was not considered appropriate to publish on this matter.

Finally, your statement that the WHO has "pulled out" may leave a false impression with your readers. The original agreement between the government of India and the WHO establishing the research unit was for a period of six years, which expired on June 30, 1975. The unit developed much essential methodology, carried out several small scale field trials and assisted in the creation of a core group of Indian scientists fully conversant with all the aspects of the research. What is left to be done is to carry out

large scale feasibility studies of new vector control methodology in areas of southern India endemic for mosquito-borne diseases, which does not require the assistance of full-time WHO staff members. It is anticipated that this work will be carried out under Indian leadership now that the WHO has handed over the unit to the Indian Council of Medical Research on the appointed date, with continued WHO technical advice and assistance if requested.

Yours faithfully,

F. J. TOMICHE

World Health Organisation, Geneva

SIR,—Your leader "Oh, New Delhi; Oh, Geneva" (July 31) might as well have been written by the World Health Organisation's Public Relations staff whom you hold responsible for the bad handling of the Indian press that ultimately, according to you, led to the closure of the Research Unit on Genetic Control of Mosquitoes (GCMU) in New Delhi.

The intention of this letter is not to highlight all the crucial omissions you had made (that would make the letter long) but only to correct a few statements which apparently have been taken out of the handout the GCMU had prepared in defence of its project.

You have dismissed the six-volume Stockholm International Peace Research Institute (SIPRI) series on chemical and biological warfare (CBW) in one sentence by saying that SIPRI "has reported that biological warfare (BW) could be conducted with infected mosquitoes."

The SIPRI series in fact says a lot more on entomological warfare. Like genetic control, it is also in the research stage. Our allegation that data gathered by the GCMU on mosquitoes can help BW research is supported by SIPRI which says that ecological data of mosquitos and dispersal data obtained from field trials are useful in BW.

It is well known that only female mosquitoes pick up and transmit viruses. Your categorical statement that GCMU's "work has been exclusively concerned with males" is however, incorrect. The GCMU was to have released at least 2,000 females a day at Sonapat. The male-female sexing error, as claimed by the GCMU, is 0.25% and in practice it may be higher.

Second, dealing predominantly with male mosquitoes need not be a great handicap. A specialist in mosquito ecology can obtain the dispersal pattern of females of a species of mosquito by extrapolating the dispersal data of males.

Your leader gives an erroneous impression that we were the only two "to level accusations of BW." Other witnesses who appeared before the Parliamentary Public Accounts Committee (PAC) belonged to the health and defence ministries. Of course they will not accuse themselves. But if you had carefully read the PAC report you would have noticed statements made by health officials admitting that both the GCMU project and the Jodhpur project (another WHO venture about which your leader is silent) could provide data useful in BW.

You may be "revolted" by the thought that the US government was using an intermediate organisation (WHO) to learn how to infect the Indians with yellow fever. Do you know that the USA was alleged to have used an FAO expert (an Indian) in 1970 to sabotage Cuban sugar cane research?

Do you know that a US expert working at the Indian Agricultural Research Institute (IARI) here was caught at the India-Pakistan border in 1971 with his luggage containing wheat germ plasm collected by the IARI?

It was unfortunate that articles criticising the GCMU and other projects were interpreted to be anti-American. This was never intended. The bird migration studies supported by the GCMU and the US army at the Bombay Natural History Society exemplified the casual attitude of some to foreign funded research. But many other countries have set up collaborative projects covering agriculture, livestock and the continental shelf.

Although we journalists appeared before the PAC late in the day, the same questions put to us were also put to official witnesses and they were given every opportunity to give answers.

You seem to regard the WHO pulling out from an "important" research project as bad. Bad for whom? Not for India which needs to tackle malaria now and not 40 years hence when genetic control may become viable scientifically—but not economically.

As far as filariasis is concerned, an expert report to the Indian Council of Medical Research had clearly said in 1971 that the strategy is not elimination of the vector *Culex fatigans*—but protection with drugs of the vulnerable population below the age

of 21 in the endemic regions.

Chikungunya and dengue are not major health problems in India (as they were made out to be during the GCMU controversy) and control of *Aedes aegypti* has the least priority. That dengue gives cross protection against yellow fever is so strongly established (Theiler, Max, *Arthropod borne diseases in vertebrates*; 1973) that grand projects to eradicate *Aedes aegypti*—even though foreign-financed and with WHO sanction—can be only anything but scientific.

Arguments that yellow fever did not strike after elimination of *Aedes aegypti* from Poona in 1953 are too weak to disprove Max Theiler's conclusion that a person immune to dengue is automatically immune to yellow fever. Such arguments are too childish to be taken seriously by the health department of any country. Yellow fever did not come to Poona perhaps because other vectors of yellow fever were also not present, or there were missing links in the epidemiological chain.

If the closing of the GCMU causes concern to British scientists they may well restart the project around London. It is better that sophisticated technologies (genetic manipulation is one) are first tried and developed in advanced countries before they are recommended to developing countries.

Your worries that India will find few opportunities to collaborate with the rest of the scientific world are unwarranted. Right now India collaborates with some 40 countries. Almost every month some science agreement or other is signed. Under such circumstances it is only natural for the PAC to recommend that foreign research in certain areas like weather modification, and oceanography should be scrutinised from the security angle.

The PAC has not banned foreign collaboration in science but has asked for the closer participation of Indian scientists who are at present given subordinate roles and not decision-making or project-management roles in foreign-funded research. The PAC has also called for thorough scrutiny by a governmental clearing body as you yourself have admitted that "almost everything has its military aspect."

We would like to ask, for instance, if the British government will allow a team of foreign scientists to go about surveying Britain's continental shelf, causing artificial earthquakes in "search of minerals", or releasing new strains of pigeons in Trafalgar Square?

C. RAGHAVAN

K. S. JAYARAMAN

Press Trust of India Ltd.
New Delhi, India

Unwelcome error

SIR,—Something went a little wrong, I am afraid, in the box headed "Wellcome Foundation" in your guide "How Britain Runs its Science" (August 28).

The Wellcome Foundation Limited is the parent company of a British based pharmaceutical group trading as Burroughs Wellcome, Coopers and Calmick around the world. Its chairman is Mr A. A. Gray. Like any other pharmaceutical group it spends money on its own research and development.

All its shares are owned by a registered charity, The Wellcome Trust, which under the Will of Sir Henry Wellcome, who died in 1936, applies all moneys it receives, as sole shareholder, to the support of medical and allied research in universities and hospitals around the world. The Director of The Wellcome Trust is Dr P. O. Williams, and I think it is the Trust rather than the foundation that should have been featured in your guide.

The figure you gave for research grants is actually the foundation's expenditure on research and development for the year ended August 31, 1974.

Yours faithfully,

BASIL SAUNDERS

The Wellcome Foundation Ltd,
London, UK

ED—Indeed the Wellcome Trust, rather than the foundation, ought to have been featured in our guide. Before anyone starts applying for a slice of the £11.5 million which the foundation allocated to research and development last year, we had better point out that the trust's spending on research assistance (1972–74) was £1,133,463.



A hundred years ago

THE German Scientific and Medical Association was opened at Graz on the 17th inst. Lieut. Weyprecht, of the recent Austrian Arctic Expedition, made a speech deprecating all past Arctic expeditions as adventurous and valueless because they constituted an international rivalry that resulted only in giving names to some ice-bound islands. The speaker, amid general applause, expounded a new programme for making Arctic expeditions more fruitful for natural science, and to enable poorer countries to undertake such expeditions.
from *Nature*, 12, 260; September, 23, 1875

news and views

It has been little more than a year since the first publication (Olins and Olins, *Science*, **183**, 330; 1974) of electron micrographs showing the "string of beads" structure of chromatin. During this period, much other evidence has been presented (*Nature*, **254**, 651; 1975) that, at this lowest level of organisation, the DNA of chromatin is folded together with histones to form morphologically repeating units (called "bodies" or nucleosomes) involving about 190 base pairs of DNA and eight histone molecules each. We still do not know the nature of this folding—the conformation of DNA within the particle, the structure of the histone complexes which constrain the DNA, and whether or not the histone content and DNA conformation of all particles are identical. These questions are examined in several new papers.

DNA folding

Valuable information about DNA folding within the nucleosome has been obtained by chemical methods. A recent paper by Germond *et al.* (*Proc. natn. Acad. Sci. U.S.A.*, **72**, 1843; 1975) examines the properties of complexes obtained by associating histones with covalently closed circular DNA isolated from simian virus 40 (SV40). Earlier papers (Olins, *J. Cell Biol.*, **64**, 528; 1975; Oudet *et al.* *Cell*, **4**, 281; 1975) had shown that the beaded-string structure could be generated by reconstituting DNA with a mixture of each of the four histones H2A, H2B, H3 and H4. By making use of SV40 DNA in the reconstitution experiments, Germond *et al.* are now able to study the effects of nucleosomes on DNA superhelix formation. The experiments depend on the use of an "untwisting extract" isolated from Krebs II ascites cells, which contains an enzymic activity capable of removing the superhelical content of circular covalently closed DNA, leaving a relaxed product that is still a closed circle. Germond *et al.* formed a series of complexes between relaxed circular SV40 DNA and increasing quantities of a mixture of the four histones and treated the complexes with untwisting extract. When DNA was then freed of histone and its superhelical content determined, it was found that superhelix had been generated. The number of negative superhelical turns present in the DNA at the end of the experiment was equal to the number of nucleosomes per molecule observed for the corresponding

String of pearls

from Gary Felsenfeld

nucleoprotein complex in the electron microscope.

These results show that the formation of each nucleosome involves creation of one superhelical turn or its topological equivalent, but do not tell us what the actual DNA conformation is within the nucleosome, since the effect of one negative superhelical turn can also be generated by unwinding the DNA double helix one full turn, or by a series of negative and positive superhelical turns which partially cancel each other. Structural considerations place some limits on speculation, however. A single regular superhelical turn of 120 Å diameter and 110–120 Å pitch will not accommodate the amount of DNA found in the nucleosome but other superhelices can be constructed (Baldwin *et al.*, *Nature*, **253**, 245; 1975). Crick and Klug (*Nature*, **255**, 530; 1975) have proposed a class of models for the nucleosome in which the DNA, rather than being smoothly bent, is kinked at intervals in order to wrap around a core of histones. One specific model that they propose involves a 95–100° kink every 20 base pairs, generating between two and three left-handed superhelical turns in 190 base pairs. This model appears to be outside the range suggested by the results of Germond *et al.*, but it is easy to imagine variations of the model which would fit better, since the sense and pitch of the kinked supercoil are sensitive to the number of base pairs between kinks.

There is some question also about what fraction of the 190 base pair repeating DNA subunit is actually in the "bead", and what fraction is in the "string". Several investigators (Van Holde *et al.*, *Biochem. biophys. Res. Commun.*, **60**, 1365; 1974; Solner-Webb and Felsenfeld, *Biochemistry*, **14**, 2915; 1975; Axel, *Biochemistry*, **14**, 2921; 1975) interpret the electron microscopic and nuclease digestion results as indicating the presence of a string occupying perhaps 50 base pairs of the subunit. This part of the DNA, seen in the microscope as a fibre 15–20 Å in diameter, is probably not supercoiled, though it could be thinly covered by a

histone chain, perhaps wound in one of the grooves of the double helix. Noll *et al.* (*Science*, **187**, 1203; 1975) however, take the position that all of the DNA in native chromatin is wound into the nucleosome, and that the strings between nucleosomes are seen only if chromatin is subject to disruptive shearing forces. The electron microscopic studies of Griffith (*Science*, **187**, 1202; 1975) on naturally occurring SV40 DNA–histone complexes indicate that the string regions are visible at low ionic strength, but disappear from view as salt concentration is raised to 0.15 M, suggesting that these regions are in some way a unique part of the unit structure.

Arrangement of histones

The other half of the nucleosome structure problem has to do with the arrangement of the histones. The known histone content of chromatin is approximately consistent (Kornberg, *Science*, **184**, 868; 1975) with a repeating unit containing eight histones (two each of H2A, H2B, H3 and H4) for each DNA repeat of 190 base pairs. An (H3H4)₂ tetramer is present in histone fractions prepared by salt dissociation of chromatin (Kornberg, *loc. cit.* Kornberg and Thomas, *Science*, **184**, 865; 1974). Kornberg proposed that the chromatin structure was built upon a core of this tetramer, to which were added two H2A–H2B dimers to complete the octameric structure. Since histones H3 and H4 spontaneously form dimers and tetramers in solution (Roark *et al.*, *Biochem. biophys. Res. Commun.*, **59**, 542; 1974; D'Anna and Isenberg, *Biochemistry*, **13**, 4992; 1974) the presence of such complexes after release from DNA does not constitute conclusive proof of their arrangement within the chromatin structure. A more satisfactory approach to the problem is to treat chromatin with protein crosslinking reagents, and identify the histone oligomers which result, a method that has proved valuable in the study of other nucleoprotein complexes. Martinson and McCarthy (*Biochemistry*, **14**, 1073; 1975) have used tetranitromethane to demonstrate the presence of an H2B–H4 dimer in chromatin; they find that this dimer also forms when histones are reconstituted with DNA, but only when H2A is also present. VanLente *et al.* (*Cell*, **5**, 45; 1975) have treated nuclei and chromatin with formaldehyde, producing dimers of H2B with H4, and H2A with

H2B, while Bonner and Pollard (*Biochem. biophys. Res. Commun.*, **64**, 282; 1975) have generated an H3-H4 dimer using carbodiimide. Thomas and Kornberg (*Proc. natn. Acad. Sci. U.S.A.*, **72**, 2626; 1975) are the first to report the isolation of cross-linked histone octamers. Chromatin is treated with dimethyl suberimidate (Me₂Sub) or dithiobis (succinimidyl propionate) (Lomant's reagent). At pH 9, reaction with Me₂Sub produces a series of protein oligomers ranging in molecular weight from dimer to octamer, but there is little or no oligomer of larger size. Using Lomant's reagent, the principal low molecular weight product is octamer, but higher molecular weight products (16-mer and higher) also occur. It is not yet determined whether the octamer represents a single species or a mixture of several complexes varying in histone content, but of about the same molecular weight. Examination of the dimers produced as reaction intermediates by Me₂Sub has led to identification of (H3)₂, H3-H4, H2B-H4, H2A-H4, and probably H2A-H2B. The same pairs were obtained when chromatin monomers (isolated single nucleosomes) were crosslinked, suggesting that these pairs are representative of short-range interactions within the monomer.

Are all nucleosomes identical in histone content and arrangement? That

is the simplest and most reasonable working hypothesis, but it remains to be demonstrated. If there is some variation in the histone content of nucleosomes, it must be subject to certain rules of substitution, since nucleosomes seem to be fairly homogeneous in protein mass, and there is some variation among histones in molecular weight. If the nucleosomes are all identical in histone content, it is still possible that variations in internal folding or arrangement exist. This is an unattractive hypothesis because multimeric protein complexes tend to have unique arrangements. On the other hand, naturally occurring histone modifications such as phosphorylation are known to have a major effect on physical properties that might affect the way histones fold or interact.

An attempt to probe the internal arrangement of histones has been made by Weintraub (*Proc. natn. Acad. Sci. U.S.A.*, **72**, 1212; 1975) who has examined the products obtained when chromatin is digested extensively with staphylococcal nuclease, and then with trypsin. The trypsin digestion removes only 20 to 30 amino acid residues from the histone N-terminals; when the resulting nucleoprotein fragments are examined by electrophoresis on acrylamide gels, eight discrete nucleoprotein bands are observed. Four of these can be shown to carry C-terminal

portions of histones H2A, H2B, H3 and H4, one carries only H3 and H4 C-terminals, and three bands are altogether free of protein, presumably because they carried the trypsin-sensitive N-terminals. If chromatin is digested first with trypsin and then with nuclease, results consistent with this hypothesis are obtained: the protein-free bands generated by nuclease-trypsin treatment are not present in the trypsin-nuclease digest because the N-terminal attachments which protect these bands against nuclease attack have been destroyed. Weintraub's results reflect a well-defined architecture of histone-DNA attachment sites within the nucleosome. But the number and size of the fragments is too large to have arisen from a single kind of nucleosome particle undergoing degradation along a single pathway. It is possible that there are several paths of degradation leading from the same starting point to different sets of products, so that the appearance of multiple fragments in the digest cannot be taken as unequivocal evidence for heterogeneity of nucleosomes. Proof that there is only one kind of nucleosome, however, also awaits definitive quantitative experiments. If nucleosomes are homogeneous, the hope exists that they can be crystallised, and their structure determined by the direct methods of X-ray diffraction. □

Yet another satellite of Jupiter has been discovered, the thirteenth. Unlike new comets and asteroids, which seem to pop up at frequent intervals, new satellites are much less common, Jupiter XII being found in 1951 and Saturn X in 1966. Needless to say planetary discoveries are rarer still, coming at a rate of about one every 75 years (Uranus 1781, Neptune 1846 and Pluto 1930).

The discovery, observations and attempts to determine the orbit of Jupiter XIII are discussed in a recent paper by four American astronomers Kowal, Aksnes, Marsden and Roemer (*Astr. J.*, **80**, 460; 1975). They used the 122-cm Schmidt telescope at Palomar Mountain and on three consecutive nights during September 1974 photographed a 6°×6° field centred on Jupiter. The plates were exposed for 120 min, the telescope being guided to follow Jupiter. At the time Jupiter was near opposition (at its closest to Earth, about 3.98 AU away) and of magnitude -2.5. The authors estimated that the faintest images that could be detected on each plate, for an object sharing Jupiter's motion, were of magnitude about +0.22, equivalent to a brightness 6×10⁹ times less than Jupiter's.

The satellite was discovered during

the course of 'blinking' the plates—a process in which two plates are looked at in quick succession and any image which has moved relative to the fixed background stars appears to jump to and fro as each plate is successively observed. Accurate measurements of the first three plates showed that the object's motion during September 11–13 could lie on two possible orbits, a direct joviocentric one and a heliocentric orbit similar to that of an asteroid. This confusion was only resolved after seven more plates were taken, the latest on December 12 when Jupiter was more than three

Jupiter XIII

from David W. Hughes

months past opposition and at a distance of 5.03 AU. Four of these were obtained using an image tube attached to the Steward Observatory's 229-cm reflector on Kitt Peak, the plates requiring only 1.5 min exposures.

Just considering their distances from Jupiter, the Jovian satellites seem to fall into three distinct groups, (I-V), (VI, VII and X) and VIII, IX, XI and XII, these being within 1.8, around 12 and between 21 and 24 million km from the planet with wide

empty gaps in between. The new satellite XIII is a member of the second group having an orbit with semi-major axis 0.074 AU (11.1 million km), eccentricity 0.147, inclination 26.7° and period 239 d. Its photographic mean magnitude is close to 21, about 1.5 magnitudes fainter than Jupiter XII. The radius of Jupiter XII is estimated to be around 8 km so Jupiter XIII is probably smaller. Whether these outer satellites are true-born children of Jupiter, formed in the same epoch, or whether they have been adopted at some later date remains as yet uncertain. Jupiter's seven outer satellites all have radii less than 12 km and could easily be captured asteroids probably with shapes and surface features like Phobos and Deimos, the moons of Mars.

Interestingly, Kowal *et al.* state that for the next few years there is about an even chance that each annual Jovian opposition could see the discovery of a new satellite, of comparable brightness to Jupiter XIII. The satellite would however have to be near its extreme elongation east or west of Jupiter. The opposition in 1975 (around October 13) is especially favourable as Jupiter is at the perihelion of its orbit and only 3.96 AU from the Sun.

From nuclear β decay to the charges of quarks

from R. J. Blin-Stoyle

ELEMENTARY particle or high energy physics became established as a subject in its own right during the immediate postwar years and its varied and often surprising revelations about the fundamental structure of matter have continued unabated since then. It developed from research into the properties of atomic nuclei; but whereas most nuclear properties can be unravelled using low energy (tens of MeV) accelerators and many body theory, the creation of new particles and the study of their properties requires vastly higher energies (tens of thousands of MeV and more) and has led to the development of most elegant but continually evolving theories. Nevertheless, low energy nuclear physics continues to make important contributions to our understanding of the physics of elementary particles. This brief commentary is intended to illustrate the foregoing by taking as an example some recent deductions of Wilkinson (published in this issue of *Nature*, page 189) based on very careful measurements of nuclear β -decay rates and energies.

Quark-parton model

To set the scene it must first be stated that research in high energy physics has led to the discovery of many hundreds of elementary particles and to the elucidation of their interactions and properties. In particular it has become clear that their behaviour is conditioned by three basic interactions which, in descending order of strength are known as strong, electromagnetic and weak. The strong interaction is responsible, for example, for the powerful forces that hold the nucleus together; the electromagnetic interaction is well known and manifests itself in all electromagnetic phenomena; the weak interaction is responsible, among other things, for the radioactive decay of elementary particles and, in particular, for the process of nuclear β decay. But the main properties of particles are naturally determined by the dominating strong interaction and at the present time it seems likely that the nature of the strong interaction is such that particles which experience it, and this includes the vast majority, are all, in some sense, composite structures of a very small set of basic particles (or their anti-particles) called quarks. For example, the component particles of the nucleus, namely neutrons and protons, are regarded as consisting of three quarks which, incidentally, may further be identified with the point-like objects known as partons indicated by some

experiments to be constituents of elementary particles.

What Wilkinson has done is to assume the correctness of the quark-parton model and then to derive the charge of these constituent particles from β -decay data. This is a remarkable connection to make and inevitably intermediary assumptions and hypotheses have to be made in achieving it. But what is important is not so much the result and the extent to which it can be considered reliable, but the fact that the connection can be made and can clearly be improved and developed in the future.

Briefly the steps taken are as follows. They start naturally from basic input experimental data—the lifetimes and energy release of certain particularly simple nuclear β decays known as superallowed transitions. The measurements involved which, it must be stressed, have been carried out to extremely high accuracy, have been the preoccupation of Joan Freeman and her colleagues at Harwell over the last decade or so and more recently by other researchers in Canada and the USA. From these data it is then possible to deduce the strength of the β -decay interaction. Fortunately the deduction is very largely independent of the nuclear structure details for the decays concerned—indeed, this is why they were chosen for study. The strength is measured by a coupling constant $g_{\beta V}$ (where V signifies that we are dealing with the vector part of the β -decay interaction), which has a similar role to the proton charge e characterising the strength of the electromagnetic interaction. In fact, the similarity is more than trivial and in deducing the value of $g_{\beta V}$ it is assumed that the weak ‘current’ responsible for the superallowed β decays is conserved (the well established conserved vector current hypothesis) in analogy to the electromagnetic current. The only difference is that it is a charged current and causes the decaying nucleus to change its charge when it decays (since it emits an electron or positron) whereas the electromagnetic current is neutral so that a nucleus decaying electromagnetically retains its charge (since it emits a photon).

Cabibbo universality

Now it is a further hypothesis of weak interaction physics that it is universal in the sense that all weak interaction processes are governed by essentially the same coupling constant g to which $g_{\beta V}$ must therefore be related. The hypothesised relation is simple, namely, $g_{\beta V} = g \cos \theta_c$ where

θ_c is known as the Cabibbo angle. Here it should be remarked that nuclear β decay conserves ‘hypercharge’, a quantity rather analogous to ordinary electric charge, but that in those decays of elementary particles which do not conserve it the relevant coupling constant is $g \sin \theta_c$. Thus, this ‘Cabibbo universality’ simply involves the sharing of the strength g between these two different categories of β decay. The value of θ_c can be determined with quite reasonable accuracy from data on hypercharge non-conserving β decays and so, using the expression $g_{\beta V} = g \cos \theta_c$ and knowing $g_{\beta V}$ and θ_c , the value of g can be obtained.

However, assuming the universality of the weak interaction already referred to, g is also the coupling constant governing the decay rate of the μ -meson into an electron, neutrino and antineutrino. This decay is unique and simple to treat theoretically since, in contradistinction to the β -decay situation, none of the particles involved experience strong interactions. In addition its rate has been measured accurately so that a value for g can be obtained very straightforwardly. However, before comparing the values of g from β - and μ -decay data, corrections due to electromagnetic effects have to be made to both values. These are known as electromagnetic radiative corrections and result from the modification of the decay rates due to the electromagnetic interactions between the particles involved in the decays. The difference in these corrections for the two decays, and this is all that concerns us here, is related to two quantities. First, being an electromagnetic effect, the β -decay radiative correction naturally depends on the charges of the constituent quarks of the neutrons and protons involved in the decay. Second, for both β - and μ -decay the correction depends on a characteristic mass M , and here we come to the final hypothesis to be introduced.

Over the last few years theories have been developed which unify the weak and electromagnetic interactions. One particularly successful version is that of Salam and Weinberg and is well supported by recent neutrino scattering experiments which establish the presence of neutral weak currents (as distinct from the charged currents already mentioned). Just as the neutral electromagnetic current couples to the photon, so the neutral weak current couples to a very heavy uncharged (that is, neutral) meson whose mass can be determined from the neutrino

scattering experiments. Assuming the validity of the Salam-Weinberg theory it then transpires that the mass M arising in the β - and μ -decay electromagnetic radiative corrections is just that of the neutral meson.

The connection is now complete and all that remains is to choose the quark charges so that the values of g derived independently from the β - and μ -decay data agree with one another. Suffice it to say that Wilkinson's results are consistent with one third integral charges for quarks as originally suggested by Gell-Mann and Ne'eman and not with integral charges as suggested recently.

As stressed earlier in this commentary the result, although extremely interesting in itself, does rest on a rather complex edifice of hypotheses

and theories some components of which are perhaps a little delicate. What is important is that it has been shown how quite fundamental information about properties of elementary particles can be derived from very accurate measurements within the framework of conventional low energy physics. There are other properties of elementary particles and their interactions that are being studied in similar ways (such as time reversal invariance, parity violating nuclear forces, exotic interactions) and there are those of us who feel that, quite apart from achieving a full understanding of the nucleus as a many-body system, there are these other important reasons for continuing detailed studies of nuclear phenomena. □

F_c receptors and Ia antigens: a postscript

from Robert S. Kerbel

Is there a unique relationship between Ia antigens and F_c receptors on the surface of B lymphocytes? As discussed in a recent *News and Views* article (*Nature*, **255**, 576; 1975), an affirmative answer to this question would almost certainly have far-reaching implications for various aspects of the immune response and its regulation: these have been succinctly summarised (*Transplant Rev.*, **23**, 159; 1975) by the same investigators who in fact first presented evidence for an identity or close association between F_c receptors and Ia antigens on B cells (Dickler and Sachs, *J. exp. Med.*, **140**, 779; 1974).

To summarise briefly: Ia antigens are a system of alloantigens controlled by the I (immune response) region of the major histocompatibility gene complex (H-2) in the mouse. They are expressed in quantitatively significant amounts on B cells, and appear to be responsible for, among other things, the generation of mixed lymphocyte responses in culture and the generation of *in vivo* graft-versus-host responses. The F_c receptor is a cell-surface component which interacts with the F_c portion of certain immunoglobulins and is found on a wide variety of cells, including B lymphocytes. It has potential significance in a spectrum of immunological phenomena. Dickler and Sachs suggested that a special relationship may exist between Ia antigens and F_c receptors on the basis of experiments in which the binding of aggregated immunoglobulin (Ig) to B cell F_c receptors was inhibited by anti-Ia antibodies but not by anti-H2K, anti-H2D, or anti-Ig antibodies.

Schirmmacher *et al.* (*J. exp. Med.*, **141**, 1201; 1975) however, found no evidence for a unique association, on

the basis of results obtained from two alternative assays both of which utilised the same basic idea of assessing the relationship of cell surface components to F_c receptors by the ability of antibody (against these components) to inhibit F_c receptor binding. As was pointed out, these results, although they provided no support for a unique association theory, did not formally disprove the notion either. More direct evidence is needed to settle this controversy: in this issue of *Nature*, Rask, Klareskog, Ostberg and Peterson (page 231) have tried to provide such evidence by isolating B cell F_c receptors and examining some of their properties. Their results appear to show that neither an identity nor close association exists between F_c receptors and Ia antigens.

Rask *et al.* prepared soluble macromolecules from crude membrane fractions of mouse spleen cells which were then subjected to affinity chromatography on a column of sepharose beads to which heat-aggregated IgG molecules had been covalently attached. Any soluble F_c receptors would be expected to bind to the aggregated Ig which could then be eluted. When such eluted material was subjected to polyacrylamide gel electrophoresis, Rask *et al.* found three types of sialic-acid containing polypeptide chains with apparent molecular weights of about 65,000, 18,000, and 15,000. Evidence was cited by the authors which indicated the three components behaved like F_c receptors in that they had affinity for soluble immune complexes.

The remainder of the experiments involved the use of rabbit antisera raised separately against the 65,000 dalton material and the mixture of the 18,000 and 15,000 dalton components.

When these antisera were incubated with mouse lymphoid cells they appeared to react only with B cells which would be expected if the antibodies had specificity for F_c receptors. IgG and F_{(ab')₂} fractions of these antisera also possessed the ability to inhibit the uptake of aggregated human IgG on to B cells.

The authors then examined the possibility of a close relationship existing between Ia antigens and F_c receptors by use of the so-called 'lysostripping' method, which is based on the assumption that independent molecules float freely in the cell membrane, and can be redistributed by antibodies into 'caps' on the cell from an originally diffuse or patchy distribution. The cells then become specifically resistant to complement-dependent lysis when further incubated with antibodies directed against the capped antigens. Antibodies against other cell surface components however retain their full cytotoxic ability provided an intimate relationship does not exist between them and the capped antigens. If such a relationship does exist, these other surface components can be 'co-capped' as well, resulting in resistance to complement-dependent lysis. The principle of co-capping of cell-surface components was one of the first methods used, for example, to show that a close physical relationship exists between histocompatibility antigens and β -2 microglobulin (Poulik *et al.*, *Science*, **182**, 1352; 1973).

When Rask *et al.* incubated spleen B cells with their presumptive anti-F_c receptor antisera so as to induce capping, the cells subsequently became resistant to lysis mediated by the same antisera; but when tested with anti-Ia sera, the cells were apparently killed, that is the Ia antigens did not appear to co-cap with the F_c receptors. The authors conclude that no unique association exists between Ia antigens and F_c receptors although they may lie adjacent to one another in the cell membrane.

An attempt to help resolve another controversy surrounding Ia antigens; their presence or absence on T cells, also appears in this issue of *Nature* (page 230) by Goding, White and Marchalonis. Previous reports have indicated the presence of Ia antigens only on B cells, or on both B and T cells. The reasons for the discrepancies are unclear but may be partially due to the nature and potency of the anti-Ia sera used in various studies. In any case, Goding *et al.* demonstrate the specific immunological precipitation of small amounts of Ia antigens from extracts of surface radioiodinated thymocytes using an anti-Ia serum of very high titre. The results are consistent with recent serological data showing the

presence of very small amounts of Ia antigens on less than 50% of thymocytes, a finding made possible by the use of a fluorescence-activated electronic cell sorter (Fathman *et al.*, *J. Immunol.*, **115**, 584; 1975). More conventional procedures such as immunofluorescence, cytotoxicity, and absorption of antisera, do not allow detection of Ia antigens on thymocytes although they readily do so on B cells.

The presence of small amounts of Ia antigens on thymocytes is more than vaguely similar to the story of Ig on thymocytes and T cells. Since it seems that the presence of Ig on T cells is sometimes a result of its passive adsorption on to the surfaces of the cells, and not to its endogenous synthesis, an obvious point arises as to whether the same is true for Ia antigens and thymocytes. Indeed, it has been demonstrated by Vitetta *et al.* (*J. Immunogenet.*, **1**, 82; 1974) that, in contrast to H2 antigens, soluble Ia antigens are readily shed into incubation medium containing Ia-positive lymphocytes. In addition, passage of Ia-positive lymphocytes down a column of nylon wool (a procedure used to deplete cell populations of B cells) seems to remove selectively some of the Ia antigens from the surface of the B cells. (Schultz *et al.*, *Cell. Immunol.*, **16**, 125; 1975, and Fathman *et al.*, *J. Immunol.*, **115**, 584; 1975).

These intriguing findings, besides being of some significance themselves with respect to Ia antigen function, will no doubt ensure perpetuation of the controversy surrounding the presence and relationship of Ia antigens to T cells. □

More new particles

In the article 'More new particles' which appeared in News and Views last week an editorial change implied that a section was a full account of work discussed at the recent Lepton-Photon conference at Stanford; the article was in fact received before the Stanford conference.

Nuclear states of high spin

from P. E. Hodgson

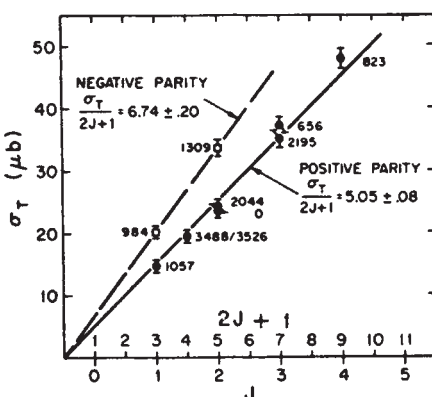
ONE of the most powerful ways of determining the spins of nuclear states is by one-nucleon transfer reactions: the angular distribution of the emitted particles is characteristic of the angular momentum transfer, and this usually suffices, sometimes in association with other data, to fix the spin of the final state in the residual nucleus.

This method works best for low

spins, corresponding to angular momentum transfers of 0, 1 and 2. For higher spins the angular distribution is not so characteristic, and the reaction may be forbidden by the spin selection rules.

Among the methods applicable to high spin states the ($^7\text{Li}, p$) reaction is proving useful, and a recent paper by Bishop and Fortune (*Phys. Rev. Lett.*, **34**, 1350; 1975) provides a good illustration of this.

At low energies on light nuclei the ($^7\text{Li}, p$) reaction proceeds predominantly through the compound nucleus. The ^7Li is captured by the target nucleus to form a compound system which then can decay by proton emission. It is then found that the total cross section for the reaction is closely proportional to $(2J+1)$, where J is the spin of the final nuclear state. This relation has been tested for a large number of states, and can be used with some confidence to determine unknown spins.



Total cross sections for the $^{14}\text{N}(^7\text{Li}, p)^{20}\text{F}$ reaction to a number of positive and negative parity states of ^{20}F plotted as a function of $(2J+1)$.

Bishop and Fortune studied the reaction $^{14}\text{N}(^7\text{Li}, p)^{20}\text{F}$, and their results for some positive and negative parity states of ^{20}F with known spins are shown in the figure. The cross sections are closely proportional to $(2J+1)$ and the ratio of the total cross section to $(2J+1)$ is 5.05 ± 0.08 for the positive parity states and 6.74 ± 0.20 for the negative parity states. The proportionality is so closely followed that it is possible to use the total cross sections for the ($^7\text{Li}, p$) reaction to determine the spins of some other states. The identification can be strengthened by seeing how well the state fits into the rotational band structure of the low-lying states of ^{20}F .

The method used is simply to calculate the spin from the expression $\frac{1}{2}[(\sigma_T/5.05)-1]$ for positive parity states and $\frac{1}{2}[(\sigma_T/6.74)-1]$ for negative parity

states and to compare these spin values with those of the possible states that fit into the band structure.

For the state at 2.87 MeV for example, the calculated spins are 3.5 for a positive parity state and 2.5 for negative parity. For the known band structure the assignment 3^- is considered the most likely. In a similar way one of two states at 2.97 MeV is probably identified as 4^- . Probable spin assignments are made to several other states as well.

This work shows the usefulness of the ($^7\text{Li}, p$) reaction in determining the spins of nuclear states, when used in conjunction with other techniques. □

Bird song dialects

from John R. Krebs

SONG birds, like humans, have local dialects. Regional variations in the form of territorial song have been found in such birds as chaffinches, tree-creeper, cardinals, white-crowned sparrows and song sparrows. Often dialect regions are quite small, in the order of a few miles across, and the transition between them may be very sudden. Within a dialect region all males have similar songs whereas between regions the song varies considerably. Playback experiments in which recorded songs are broadcast inside a territory have shown that males can tell the difference between songs of their own dialect and those from other regions (Lemon, *Anim. Behav.*, **15**, 538-595; 1967; Milligan and Verner, *Condor*, **73**, 208-213; 1971); territorial males react more vigorously to songs of their own dialect region.

Dialects probably originate as a result of young birds learning the songs of nearby adults soon after leaving the nest. In the white-crowned sparrow, for example, young males learn the territorial song within the first three months after leaving the nest, so that they are likely to learn the characteristic song of their birthplace.

Several workers have suggested that dialects play a role in the genetic adaptation of populations to local environmental conditions, by acting as a reproductive barrier between populations. This is based on the assumption, as yet untested, that females as well as males can recognise songs of their own dialect region, and will mate preferentially with local birds. Nottebohm (*Condor*, **71**, 299-315; 1969) found that dialect boundaries in a South American sparrow, the chingolo, correspond with changes in habitat structure, for example from grassland to forest, which is what one would expect if dialect regions represented locally

adapted populations. The most obvious prediction of this hypothesis is that dialect boundaries should coincide with genetic boundaries between populations, and this has recently been tested by Baker (*Evolution*, **29**, 226-241; 1975). Baker, working with white-crowned sparrows, used electrophoretic techniques to examine the differences between local populations in the frequency of marker genes, in the Colorado Rockies and at Point Reyes, California. The study area in California was apparently uniform open scrubland, but nevertheless there were sharp dialect boundaries associated with minor habitat features such as an old fence or a ditch. Baker examined the changes in allele frequency across one dialect boundary, and the overall picture for six loci was of quite marked changes at the boundary, with little change within either dialect region. This result seems to support the reproductive isolation hypothesis, but if the populations are adapted to local conditions, the adaptations must be rather subtle since the habitat did not obviously change at the boundary.

In the East River valley system in Colorado, Baker found that all the birds sang the same dialect, but there were changes in allele frequency at three loci associated with changes in altitude. So genetic subdivision of populations can occur without division into dialect regions. Taken together, Baker's results do not provide convincing support for the role of dialects in reproductive separation of populations, and he suggests that whether or not dialect and genetic boundaries coincide may depend on how an area is colonised. The Californian scrub habitat is occasionally devastated by fire, so that the white-crowned sparrow population is reduced to a few birds living in isolated patches of surviving scrub. Dialect regions probably arise through the subsequent spreading of these isolated groups as the habitat recovers, slight differences in song between the founder members of each group being perpetuated by cultural transmission, and slight genetic differences arising by chance. The Colorado valleys are stable and the population of sparrows does not get separated into isolated groups, hence dialect regions have not been established. Presumably genetic changes have arisen through selection in the face of gene flow.

The next step in following up Baker's study will be to find cases (for example small islands) where the colonisation and establishment of dialect differences can be tracked in detail. It remains to be seen whether or not a clearer link between genetic and dialect subdivisions will be found in species like the chingolo where dialects are associated with habitats. □

Genetic arrangement in eukaryotic DNA

from T. H. Rabbitts

THE amount of DNA in eukaryotic genomes is known to be extremely large. The mammalian genome, for example, consists of around 5×10^9 base pairs (per haploid set) which would be sufficient to code for about two million proteins each containing 650 amino acids. In addition it is known that eukaryotic DNA consists of both single copy sequences and repetitive sequences (sequences present as multiple copies in the genome). Two questions currently receiving critical appraisal are how the different types of DNA sequences are arranged in the genome and how much of the genome actually carries protein coding information.

Much evidence has been accumulating in support of the general concept that in many eukaryotes a large percentage of the middle repetitive DNA (DNA sequences present in a few hundred copies per haploid genome) is interspersed amongst single-copy sequences. These single copy sequences are typically about 1,500 nucleotides long with the repetitive elements being around 300 nucleotides long. In two recent papers, Britten, Davidson and their co-workers have extended this evidence and produced a general review of DNA sequence organisation of eukaryotes. In the study by Goldberg *et al.* (*Chromosoma*, **51**, 225; 1975) the sequence arrangement of five marine invertebrates was determined by the examination of the reassociation kinetics of two different length DNA fragments (300 and 3,000 bases). Hybrid formation was assayed by hydroxyapatite fractionation so that DNA fragments carrying a repetitive sequence plus a non-repetitive sequence would kinetically appear in the repetitive part of the DNA reassociation curve. Using this method, all the genomes studied were found to possess a large portion of their single copy sequences interspersed with repeating sequences. In addition it was shown that these repetitive sequences are about 300 bases long.

Davidson *et al.* (*Chromosoma*, **51**, 253; 1975) have correlated the data on sequence organisation in a wide variety of metazoa. They conclude that the DNA of organisms from most branches of the evolutionary tree shares the property of containing both unique and repetitive sequences in at least 70% of DNA fragments 2,000 to 3,000 bases long. The only exception (for which data are available at present) seems to

be *Drosophila* DNA. A study of this DNA has been made by Manning *et al.* (*Cell* **4**, 141; 1975). These workers examined the interspersion pattern of single copy and repeating sequences using both the hydroxyapatite procedures and electron microscopy of re-associated fragments of differing length. It was observed that *Drosophila* DNA has an average distance of around 13,000 nucleotides between middle repetitive sequences. This number of bases is on average about six times as great as that found in the organisms reviewed by Davidson *et al.* In addition to the data of Manning *et al.*, randomly chosen fragments of *Drosophila* DNA have been cloned in *E. coli* and the re-association properties studied (Wensink *et al.*, *Cell*, **3**, 315; 1974; Glover *et al.*, *Cell*, **5**, 149; 1975). So far, the cloned DNA chosen for study has revealed only segments of DNA lacking internal repetition over as many as 15,000 to 27,000 bases. These data seem to prove that *Drosophila* DNA does not possess short repeating sequences interspersed with single-copy DNA. Further examples of the '*Drosophila*' type organisation may well soon be discovered.

Whether the interspersing nature of repetitive sequences in organisms such as *Xenopus* has any direct relevance to gene control is far from clear. Indeed, there are indications that not all repeating DNAs possess regulatory function since cytoplasmic messenger RNA-like molecules have been shown to hybridise to repetitive sequences in a variety of organisms. This leads to the question of how much of the genome actually carries protein coding information. Hybridisation kinetic analysis has recently been used to examine this problem quantitatively. Bishop *et al.* (*Nature*, **250**, 199-204; 1974) prepared complementary DNA (cDNA) against poly(A)-containing RNA of HeLa cell cytoplasm using reverse transcriptase. The kinetic complexity of the RNA population was then determined by hybridisation of excess RNA with cDNA. Using this method, Bishop *et al.* concluded that HeLa cell cytoplasm contains as many as 35,000 different mRNA species. This number of gene products is much higher than the number obtained from a similar study of *Drosophila* referred to by Izquierdo and Bishop and described in detail by Levy and McCarthy (*Biochemistry*, **14**, 2440; 1975). In the latter cases, an mRNA complexity around 4,000 to 7,000 species was found. These numbers compare nicely with the number of bands seen by Bridges in polytene chromosomes (*J. Hered.*, **29**, 11; 1938). What then is the function of the excess DNA which makes up the genome?

Excluding the genes for the rRNAs and tRNAs it begins to look as if a much greater complexity of nuclear

RNA exists than is found for cytoplasmic RNA. Hough *et al.* (*Cell*, **5**, 291; 1975) have studied the hybridisation of purified radioactive unique sea urchin DNA in RNA-excess reactions with gastrula nuclear RNA. These experiments indicate that around 28% of the total complexity of the sea urchin genome is represented in this nuclear RNA. This complexity of nuclear RNA is at least an order of magnitude higher than that found for sea urchin polysomal mRNA. Earlier experiments (Getz *et al.*, *Cell*, **4**, 121; 1975) with mouse Friend cells arrived at similar general conclusions. In this work, the complexity of poly(A)-containing nuclear RNA was assessed by kinetic hybridisations with cDNA. The authors conclude that at least five times more unique DNA is represented in the nuclear RNA than in the polysomal mRNA of these cells. Although the numbers involved in the papers of Hough *et al.* and Getz *et al.* are somewhat different (this difference probably being a result of the use of total nuclear RNA in the former case and poly(A)-containing nuclear RNA in the latter case), the general conclusion is the same—a larger amount of the genomic information is synthesised into nuclear RNA than is present in the cytoplasmic poly(A)-containing RNA. This disparity between the two RNA populations allows speculation regarding the function of the RNA which seems to remain in the nucleus.

Even with these detailed numerical analyses of eukaryotic gene complexity, very little is known of the fine structure of individual genes and genetic units. Studies of these structures should clarify the nature of the controlling elements and specific informational organisation in eukaryotic DNA. □

Excited atoms

from a Correspondent

The ninth international conference on "The Physics of Electronic and Atomic Collisions" was held in Seattle on July 24–30.

ATOMIC physics is finding applications in horticulture: Sir Harrie Massey, delivering the opening scientific talk of the conference, reminded delegates that the study of negative ions, in what was non-applied research in atomic physics, resulted in the method for determining levels of DDT in crops. Though that particular spin-off was successful the nuclear fusion story is a less cheerful one: the level of aid so far given to the thermonuclear fusion programme is worrying. Delegates at

the conference were aware of the immediate need for atomic scattering cross-section data to assist in the construction of a viable fusion machine. It was stressed that measured cross sections for electron scattering of highly excited and ionised atoms and molecules was needed immediately to enable a composite picture of the competing processes in the 'fusion plasma' to be made. An estimated 40% of the power losses from the injected ion beams occurs through the presence of impurity ions such as C^+ , Fe^+ , Mo^+ by way of electron capture, dielectronic recombination and bremsstrahlung losses. But the problem, as expressed by some delegates, is that "the cross section we want today isn't the one we need tomorrow".

Experimental investigations into highly excited states of atoms, for example H, Na, Xe, has advanced very rapidly since the last meeting. In addition to using charge exchange methods for producing high rydberg states, one group (R. F. Stebbings *et al.*, Rice University, Houston) reported that they had used tunable dye lasers in selecting individual high rydberg states in Xe and then measured the ionisation cross section in SF_6 . The use of these highly excited atoms in scattering experiments is now within the range of existing experimental techniques.

Lasers have not yet been exploited to the full in atomic physics for state selection. State and velocity selection of neutral atoms by photo-detachment of the negative ion (C. Linberger, GILA, Boulder, Colorado) is sure to advance the field of reactive scattering. Several groups are considering the determination of atomic beam composition by selective laser pumping.

Doppler free spectroscopy has advanced rapidly; fine and hyperfine transitions can now be measured more easily and accurately than before. The excitation of the $H(1S) \rightarrow H(2S)$ transition by two 2,341 Å photons, using the $H\beta$ level as a reference, has enabled the Lamb shift of $H(1S)$ to be measured. Multiphoton processes and level shifts using lasers are being extensively investigated in many laboratories in the USA and Europe.

The excellent electron scattering work done by many groups has now been complemented by positron scattering data. The measurement of positron differential scattering cross sections awaits the development of higher intensity sources.

The conference indicated very strongly the need now for experimental data on excited state phenomena, not only to aid applied research, but in order to understand better the nature of continuum interactions (U. Fano, University of Chicago) and the formation of bounding molecular states. □

Progress in binary circles

from John Faulkner

At three-year intervals since 1969, the International Astronomical Union has sponsored meetings to review some aspect of our knowledge of close binary stars. The latest and by general consent most successful meeting took place in and around the newly designated Hoyle building at the Cambridge Institute of Astronomy on July 28–August 1. Attendance has more than doubled at successive meetings; IAU Symposium No 73, "The Structure and Evolution of Close Binary Systems", attracted over 110 participants.

ONE reason for the rejuvenation of the subject has been the realisation that most galactic X-ray sources indeed consist of such systems in relatively short lived but highly observable stages of mass transfer between components. Herb Gursky (Harvard University), enjoying his role of Grand Old Man of X-ray astronomy, remarked on the new found respectability of his subject in being awarded the opening day rather than the last afternoon as before; he went on to show that while there is convincing statistical evidence that systems containing type II supernovae subsequently turn into X-ray sources, the situation is quite otherwise for type I. J. B. Hutchings (Dominion Astrophysical Observatory) reported an impressive spectroscopic confirmation of the disputed period of Sco X-1. Earlier this year Gottlieb, Wright and Liller (Harvard University) announced that a photometric study of old Harvard plates taken between 1889 and 1974 showed luminosity variations of only ~ 0.2 mag with a period of 0.787313 d. This remarkable discovery stood in serious conflict with post-X-ray discovery work by other groups and some scepticism had been expressed. However, Hutchings, Cowley and Crampton have now verified the Harvard period by studying the variable radial velocities of He II emission lines. Taken together, these investigations demonstrate the value of the classical and often lengthy programmes eschewed and frequently despised by the "black holier than thou" school of esoteric astrophysicists. Periods considerably less than a day (Cyg X-3 has $P \sim 4.8$ h) pose evolutionary problems for the picture of neutron stars orbiting more or less massive companions and both P. Ostriker (Princeton University)

and B. Paczynski (Polish Academy of Sciences, Warsaw) discussed how the systems might arise. Both required at an earlier stage that rapid mass transfer should produce a system with a neutron star orbiting the core of a distended giant star inside a common envelope. Ostriker envisaged an accretion wake to the neutron star inducing a gravitational drag on its orbit (reminiscent of Chandrasekhar's "dynamical friction"); concomitant heating blew away the envelope. Paczynski imagined the neutron star, through entrainment, acting as a giant paddle, transferring angular momentum from the motion to the common envelope. Both thought the system might at some stage resemble a planetary nebula surrounding a late-type star; whether these remarks were prompted solely by the presence of such puzzles in the literature was unclear. While obviously preliminary and exploratory, these suggestions stimulated much subsequent discussion.

B. Warner (University of Cape Town) presented an outstanding paper on observations of dwarf novae. While covering the many timescales exhibited by these objects, he emphasised the rapid periodicities ($P \sim 16\text{--}34\text{ s}$ mainly) found by himself and colleagues. Warner and E. L. Robinson (University of Texas, Austin) had previously shown that the pulsations occurred only at or after outburst maxima, a result which everyone agreed placed severe constraints on outburst models (no-one being quite clear however just what the constraints were!) Warner now seems to have found one case of pulsation on the rising branch; it is questionable whether this will be the precursor of similar discoveries or the one exception that seems to exist to every generalisation in this field. Warner announced the discovery of three new types of periodicity in these variables (including one which, as Virginia Trimble remarked, is called "drifting subpulses" in pulsars), thus retaining for these systems the record for the largest number of interesting Fourier components. Almost everyone present now seems prepared to jump on the bandwagon of accretion disk instabilities to explain the quasi-periodic outbursts of these stars; the sceptic might remark that the defects of this explanation have yet to be as thoroughly explored as those of competing mechanisms.

Martin Rees (Cambridge University) gave a typically complete and ultimately sceptical review of accretion disk lore largely in the context of compact X-ray sources, ending with at least six criticisms of generally accepted assumptions as he ran out of time. X-ray emission processes, mass transfer dynamics, evolution and possible

tests of gravitational theories were all crying for attention and getting it in this trendy subject.

The classical topic of contact binaries provided the opportunity for two young workers to impress the audience with their computational skill in following cooperative (or even sometimes uncooperative) evolution of two stars. Both R. Webbink (Cambridge University) and B. Flannery (Institute of Advanced Study, Princeton) have developed modified forms of the evolutionary code of P. P. Eggleton whose method seems uniquely suited to this kind of problem. Webbink studied unsteady mass transfer in close binaries and produced an example in which a catastrophic runaway developed. Flannery showed how contact stars of

almost equal mass evolved to a stage of essentially cyclic thermal instability and mass exchange with mass fraction oscillating between ~ 0.56 and ~ 0.62 in periods of $\sim 10^7$ yr. Such systems seem a very satisfactory explanation for a number of the WUMa "main sequence contact binaries".

All in all, the symposium was a fine blend of observational facts, theoretical explanations and sheer unbridled speculation (at one point Warner remarking that the quality of the arguments led him to conclude that the cow lowing at 2-s intervals in an adjacent field had a density of $\sim 10^8\text{ g cm}^{-3}$). Commissars Mitton and Whelan marshalled the resources of the Institute splendidly for a meeting that will long be remembered. □

Comets may conceal chemicals

from John Gribbin

Two of the more speculative of recent suggestions about the place of comets in the Galaxy have now been investigated in some detail by Fred Whipple (*Ast. J.*, **80**, 525; 1975). The ideas are that enough material may be locked up in comets to have a significant effect on the evolving chemistry of the Galaxy, and that γ -ray bursts may be produced by the impact of comets on to neutron stars. The score now seems to stand at Whipple 1, comets 1, with the first possibility still tenable but the second speculation now ruled out of order.

The gamma-ray burst hypothesis always did look a trifle implausible and seemed to require the presence of interstellar comets, since the violent events in which neutron stars are believed to form would be highly likely to disrupt any bound system of comets possessed by the original star. Even though a few comets with hyperbolic orbits have been seen, Whipple's calculations show that for four candidates—the only ones, in a sample of 89 comets with well determined parameters—the chance of any one having arrived on such an orbit from interstellar space is less than 1 in 10^4 , so that for the whole "family" to be interstellar they would be running against odds of 1 in 10^{16} . It is far more likely that the orbits were slightly hyperbolic because "of velocities imparted to solar system comets by the perturbation of passing stars, because of nongravitational effects induced by comet rotation and jet action, because of chance observational errors, or because of systematic displacements of the centre of the observed coma from the direction of the nucleus." But even without wandering through interstellar space free from their parent stars, comets can

play a part in the chemical evolution of the Galaxy. As any galaxy ages, the proportion of metals (by which astronomers mean anything except hydrogen and helium) in its stars increases through nucleosynthesis. Newly forming stars in an old galaxy contain the debris from previous stellar explosions, and do not have the same composition as newly forming stars in a young galaxy. But according to some calculations (B. M. Tinsley and A. G. W. Cameron, *Astrophys. Space Sci.*, **31**, 31; 1974) the mean metal abundance of disk population stars has grown more slowly than expected on the seemingly reasonable assumption that some 2% of the mass from one generation of stars is recycled in the next generation of star formation. Comets could provide a sink for the missing heavy metals (if any really are missing, of course), but Tinsley and Cameron need so many comets that their mass would be 25% of the total mass of gas in the interstellar medium of our Galaxy today—about 1% of the mass locked up in stars.

Rather surprisingly, perhaps, Whipple is able to show that a great mass of comets—more than 1% of the solar mass—could be gravitationally bound to the Sun in a symmetric shell just beyond the radius of the periodic comets and remain completely undetected by present techniques. This provides a lot of leeway for those theorists who wish to, to invoke the presence of such comets to explain discrepancies between their theories and observation, but will not please the more spartan souls who prefer to have well-defined limits on the parameters of the real Universe and who take great pains to fit their models within those rigid limits.

review article

Opiate receptor in normal and drug altered brain function*

Solomon H. Snyder†

Recent biochemical identification of the receptor site for pharmacological actions of opiates helps elucidate how these drugs relieve pain, elicit euphoria and 'addiction', and provides simple, direct approaches to developing potentially non-addicting analgesics. The isolation of a morphine-like peptide which may be a central nervous system neurotransmitter sheds light on normal brain mechanisms regulating pain and emotion.

EXTRACTS of the poppy plant have been used since the days of the Homeric epics medically and recreationally to relieve pain, induce sleep, ease anxiety, for diarrhoea, heart failure and simply to promote a sense of well being. It has long been known that opiates can be addictive and a major goal of opiate research is to develop a non-addictive opiate analgesic. Although non-opiate pain killers such as aspirin are useful in some situations, only opiates seem to be effective in the treatment of severe and protracted pain. Besides such a practical goal, understanding how opiates act may elucidate fundamental questions in pharmacology. Opiates provide the classic paradigm for tolerance and physical dependence, which can occur to a variety of drugs such as alcohol and barbiturates.

What opiates might tell us about normal brain function is a question that prompts consideration of the opiate receptor itself. The fairly stringent chemical requirements for opiate activity, the fact that opiate effects are highly stereospecific and that some opiates, such as etorphine, are so potent that they act in doses even lower than LSD, all suggest that opiate actions must involve highly selective sites or "receptors" in the brain. The existence of opiate antagonists also favours the receptor concept. Opiate antagonists are drugs, closely related in structure to the corresponding opiate analgesics, which may have little or no analgesic or euphoric effect themselves but which can completely reverse action of opiate analgesics. It seems as if the antagonists occupy opiate receptor sites, doing nothing themselves, but preventing access of opiate analgesics. If specific opiate receptors exist in the brain, one would assume that they were created for some normally occurring substance. Indeed, researchers have identified a normally occurring morphine-like factor in the brain with high affinity for specific opiate receptor sites. The distributions of the morphine-like factor and the opiate receptor itself correspond in part to certain pain pathways in the brain,

suggesting that the morphine-like factor may be a neurotransmitter for pathways in the brain mediating pain and emotions. A detailed understanding of such systems might help elucidate some riddles of addiction.

Demonstrating the opiate receptor

The most direct way to identify receptor sites is to measure the binding of radioactive drugs to brain tissue. Like most chemicals, however, opiates bind to almost any biological or non-biological membrane so that nonspecific binding, not associated with the receptor, greatly exceeded any receptor binding in most early studies. Goldstein *et al.*¹ enunciated the criterion that pharmacologically relevant opiate receptor binding should, like opiate analgesic effects themselves, be stereospecific.

In our laboratory, we developed simple technical manoeuvres to amplify specific receptor binding and decrease nonspecific binding. Thus, if opiates bind with considerable affinity to their receptor sites, the use of small amounts of drug labelled to high specific radioactivity favours specific, as opposed to nonspecific, binding, whereas washing tissue thoroughly but rapidly after binding preferentially removes nonspecific binding. On this basis, specific opiate receptor binding has been demonstrated to brain and guinea pig intestine²⁻⁵.

But stereospecific binding itself is not a sufficient criterion for the pharmacologically relevant opiate receptor. Some glass filters⁶ and numerous acidic lipids which may or may not be related to the biological receptor^{7,8} bind opiates stereospecifically. To ensure that binding to brain membranes represents receptor interactions that operate *in vivo*, many drugs were shown to exhibit affinity for the opiate receptor which closely parallels analgesic potency, while non-opiate drugs have negligible affinity for the opiate receptor. Ideally, one should compare pharmacological potency and receptor binding in the same system, which is feasible because the ability of opiates to inhibit electrically induced contractions of the guinea pig intestine closely parallels analgesic activity^{9,10}. Affinity of both opiate agonists and antagonists for receptor binding sites is closely similar in brain and guinea pig intestine and pharmacological and binding potencies of many opiate agonists and antagonists in the same strips of guinea pig intestine are well correlated¹¹. Besides supporting the other evidence that binding *in vitro* involves sites that mediate opiate activity

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in vivo, these observations shed light on certain basic drug receptor concepts. Some receptor theories postulate that drugs can exert maximal pharmacological responses while occupying only a small fraction of the total number of pharmacologically active receptors. If a substantial portion of opiate receptors does not normally mediate pharmacological responses, and are thus "spare receptors", then there should be major discrepancies between drug concentrations filling a given percentage of binding sites and concentrations required to produce the same percentage of a maximal pharmacological response. Opiate concentrations occupying half the binding sites in the guinea pig intestine correspond closely, however, to those eliciting half-maximal pharmacological responses, whether agonist or antagonist, which suggests that opiate effects can be explained without invoking spare receptors.

Differential receptor interactions of opiate agonists and antagonists

Opiate antagonists are important for several practical and theoretical reasons. Pure opiate antagonists, such as naloxone and naltrexone, are often life-saving antidotes to opiate overdose. Pure antagonists may also be valuable in treating heroin addicts. The notion is to administer a long-lasting pure antagonist, which will produce no analgesia and euphoria of itself, but which will prevent the effects of subsequently administered heroin. Thus deprived of its ability to elicit a 'high', heroin will cease to be attractive to the addict.

Of greater theoretical and practical importance are opiates which are mixed agonist-antagonists, since some of these agents are relatively non-addicting analgesics. The idea of combining antagonist with agonist activity to lessen the addictive potential of an opiate stems from demonstrations that the opiate antagonist nalorphine possesses analgesic, and thus "agonist" properties¹². Unfortunately, nalorphine, cyclazocine, and levallorphan, antagonists 'contaminated' with agonist activity, are not suitable for routine use in treatment of pain, because at analgesic doses they often produce anxiety, agitation, and psychotomimetic effects. Antagonists with yet more agonist activity, referred to here as mixed agonist-antagonists, have been more promising. Pentazocine, a mixed agonist-antagonist, is already clinically marketed and relieves pain with a low incidence of psychotomimetic effects and with relatively little propensity to produce physical dependence and compulsive craving. Several other mixed agonist-antagonists, while not yet in routine clinical use, offer promise as relatively non-addicting analgesics.

Traditional opiate antagonists differ chemically from agonists only in the substitution of an N-allyl, N-cyclopropyl or related group for the N-methyl of agonists. It is quite difficult to predict chemical features which result in a mixed agonist-antagonist. In our early studies of opiate receptor binding with buffer solutions lacking sodium, agonists and antagonists bound with similar affinity³. Later, we discovered that low concentrations of sodium enhance receptor binding of antagonists but decrease the binding of agonists^{13,14}. The effect of sodium is highly specific. It can be mimicked to some extent by lithium, whose atomic radius is similar to that of sodium, but not by other monovalent cations such as potassium, rubidium or caesium. Sodium seems to accelerate the rate at which antagonists bind to the receptor¹⁵ and to speed the dissociation of agonists from the receptor¹⁴. To evaluate the influence of sodium on receptor interactions of a wide range of opiate agonists and antagonists, we measured the extent to which sodium alters the ability of drugs to inhibit receptor binding of ³H-naloxone. The resultant "sodium response ratio" is the ratio of the concentration of drug which inhibits ³H-naloxone binding 50% in the presence of sodium to the

comparable concentration when sodium is omitted from the incubation medium. This sodium response ratio differentiates opiate agonists and antagonists. For pure antagonists, such as naloxone, the ratio is 1, while for a variety of opiate agonists, the ratio is between 12 and 60. Antagonists which are 'contaminated' with some agonist activity have ratios somewhat greater than 1 but less than 3. The mixed agonist-antagonists we examined have ratios between 3.3 and 6.4, clearly in an intermediate area.

Thus it seems possible to predict the pharmacological properties of different opiates simply by measuring receptor interactions in the presence and absence of sodium, affording a rapid and inexpensive screen for mixed agonist-antagonist 'non-addicting' analgesics. Chemists need not synthesise hundreds of grams of drug for screening in intact animals; a fraction of a milligram will suffice.

Model of opiate receptor function

The selective and pharmacologically relevant influence of sodium on opiate receptor binding seems an integral feature of receptor functioning. Sodium seems to increase and decrease respectively the numbers of antagonist and agonist receptors. Since opiate agonists and antagonists are so similar chemically and compete with each other for receptor binding, we postulate that both drugs bind to the same receptor but that the opiate binding site can vary as the receptor is transformed between two conformations^{14,16,17}. With normal body sodium levels, the receptor is largely in the "sodium" state for which antagonists have a selectively high affinity, whereas opiate agonist actions take place when agonists bind with their selectively high affinity for the "no-sodium" state. Antagonists block morphine actions by occupying sodium states of the receptor and shifting the equilibrium to reduce the number of no-sodium receptors available for morphine. Although agonists and antagonists have selective high affinity for no-sodium and sodium states of the receptor respectively, mixed agonist-antagonists would have similar affinities for the two forms.

The interconversion of the two forms of the opiate receptor presumably involves folding, unfolding, aggregation, disaggregation or other modifications in protein structure, since the exquisite sensitivity of receptor binding to proteolytic enzymes¹⁸ indicates that the opiate receptor is proteinaceous. One might expect protein-modifying reagents and enzymes that affect protein structure to alter the interconversion of receptor sites. Since the opiate receptor should normally be largely in the antagonist or sodium state, agents interfering with receptor interconversion should selectively reduce agonist binding. Consistent with these predictions is the observation that protein-modifying reagents and proteolytic enzymes in low concentrations do selectively reduce opiate agonist binding with negligible effects on antagonist binding¹⁹⁻²¹. The effects of protein-modifying reagents and enzymes seem to be directly related to sodium binding by the opiate receptor, for the reagents and enzymes increase the sensitivity of opiate agonist binding to inhibition by sodium.

Divalent cations may also have a role in the normal functioning of the opiate receptor. Low, physiological, concentrations of manganese and magnesium affect the opiate receptor in a fashion diametrically opposite to sodium; they selectively increase the binding of opiate agonists, by reducing receptor sensitivity to sodium²². Divalent cations act selectively; for while manganese, magnesium and nickel are active, calcium fails to enhance opiate agonist binding. The influence of chelating agents indicates that endogenous divalent cations regulate opiate receptor functions. Thus, treating brain membranes with EDTA, which chelates most divalent cations, lowers opiate agonist binding, while EGTA, which chelates calcium but not manganese and magnesium, has no influence on receptor binding.

The postulated interconversion of agonist and antagonist

conformations and the influence of sodium resemble general models for neurotransmitter receptor behaviour²³⁻²⁵. Normally receptors sites are in the antagonist or "off" conformation. Synaptic transmission occurs only when a compound binds selectively to the agonist conformation of the receptor. One or the other of the receptor states possesses a binding site for the ion whose conductance is altered in synaptic transmission. When the neurotransmitter attaches to the receptor, the binding of this ion is altered to produce the appropriate change in ionic conductance. If the endogenous morphine-like substance is a neurotransmitter, one would speculate that sodium is the ion whose conductance is changed by synaptic activity.

Direct evidence for selective interactions of an ion and receptor binding in synaptic transmission has been obtained for the influence of chloride on glycine receptor binding. Receptor sites for glycine, a major inhibitory neurotransmitter in the mammalian central nervous system, can be labelled with radioactive strychnine, a potent glycine antagonist²⁶. Chloride is the ion whose conductance is increased by the inhibitory synaptic actions of glycine. Chloride and other anions inhibit the binding of strychnine to the glycine receptor in proportion to their ability to mimic the synaptic activities of chloride²⁷.

Regional mapping

Brain functions whose distributions parallel those of opiate receptors may be implicated in opiate actions. Because opiates elicit analgesia, brain structures mediating pain are natural suspects. Sharp, prickly pain, which can be discretely localised, is conveyed by a laterally located sensory pathway with somatotopic representation in the ventrobasal thalamus among other regions. Slower, more chronic and poorly localised burning pain involves multisynaptic, slowly conducting, medially located pathways, especially the palaeospinothalamic and spinoreticulodiencephalic pathways. These lack somatotopic representation and include areas of the brain such as the periaqueductal grey, the medial nuclei of the thalamus and several parts of the limbic system²⁸.

Opiate receptor binding varies dramatically throughout the monkey and human brain^{29,30}. The amygdala binds most, but only slightly more than the periaqueductal grey of the midbrain, hypothalamus and medial thalamus. Receptor binding in the caudate nucleus is also high. Within the cerebral cortex there are marked variations: the frontal cortex for example binds more than four times the amount of opiate bound by the precentral, postcentral gyri and occipital pole. Receptor binding is very low in the spinal cord, cerebellum and white matter.

This map closely resembles the distribution of palaeospinothalamic and spinoreticulodiencephalic pain pathways, and certain of these areas, especially the periaqueductal grey, correspond to those in which implantation of morphine most effectively produces analgesia³¹. Opiate antagonists elicit withdrawal symptoms in addicted animals best when implanted in the medial thalamus, which is also very rich in opiate receptors³². The amygdala, apparently an exception not classically associated with pain pathways, may relate to affective components of pain since electrical stimulation of the amygdala does produce fight and flight reactions. Euphoric actions of morphine may be mediated by the amygdala and other areas of the emotion-regulating limbic system rich in opiate receptors.

Recent autoradiographic studies provide even more precise localisation of opiate receptor sites³³. For such studies it was first necessary for us to achieve reliable techniques for labelling receptors in the intact animals³⁴. After intravenous administration of ³H-diprenorphine, an extremely potent antagonist, 80% or more of radioactivity accumulating in the brain is associated with opiate receptor sites³³. For successful autoradiography, diffusion of the radioactive drug away from the receptor site during the fixation

process must be prevented, a difficult task with a drug such as an opiate which does not form covalent chemical linkages to tissue proteins during fixation. In our studies, fixing tissue at very low temperature prevents leakage of ³H-diprenorphine from receptor sites so that essentially all autoradiographic grains are associated with the opiate receptor³³. Grain density parallels the regional distribution of the opiate receptor as determined by dissection and biochemical assays. At a microscopic level, opiate receptor binding is even more discretely localised (Table 1). At one level of the midbrain, grains are highly concentrated over the locus coeruleus with much lower grain counts in closely adjacent nuclei. Interestingly, small doses of morphine slow firing rates of locus coeruleus cells but not adjacent cells³⁵. Since the locus coeruleus consists almost exclusively of noradrenaline cell bodies, its high density of opiate receptors may explain the numerous reports of effects of opiates on the metabolism of biogenic amines including noradrenaline³⁶. At another level of the midbrain, grains concentrate over the zona compacta of the substantia nigra, whereas the adjacent zona reticulata of the substantia nigra has very few receptor grains. The zona compacta but not the zona reticulata consists exclusively of dopamine cell bodies. Thus both major catecholamine systems on the brain are intimately related to opiate receptor sites. An association with major sensory systems in the central nervous system is indicated by the sharp band of opiate receptor grains overlying the substantia gelatinosa in the lower brain stem and spinal cord³³. The substantia gelatinosa is an important "way station" for the upward conduction of sensory information relating to pain.

Table 1 Distribution of autoradiographic grains labelling the opiate receptor in coronal sections of rat brain

Region	Autoradiographic grains per 100 μm^2
Nucleus caudatus putamen	11.5
"Streak" ventral to corpus callosum	10.5
"Clusters"	1.7
Low density areas	1.2
Fibres	10.4
Substantia gelatinosa (spinal cord)	10.0
Locus coeruleus	6.5
Amygdala medialis	5.8
Amygdala centralis	6.5
Zona compacta of substantia nigra	5.5
Thalamus medialis	5.2
Periventricular substance	5.1
Habenula	5.0
Lateral habenula	3.8
Nucleus periventricularis (of hypothalamus)	2.8
Nucleus ventromedialis (of hypothalamus)	2.1
Dentate gyrus	2.0
Motor cortex	1.9
Zona reticulata of substantia nigra	1.7
Hippocampus	0.7
Corpus callosum	0.8
Fimbria	1.3
Ventricle III	1.1
Pyramidal cortex	0.8
Cerebellum	0.8
Nucleus of cranial nerve V	0.8

These data are derived from the study of Pert, Kuhar and Snyder³³. Rats (170 g) were injected with 125 μCi of ³H-diprenorphine (13 Ci mmol^{-1}), killed at 1 h and brains rapidly cut into 3-4 mm coronal sections, placed on microtome chucks and lowered slowly into liquid nitrogen "slush". Sections of 4 μm thickness were cut at -18°C in a Harris cryostat microtome and transferred by "thaw-mounting" in the dark to slides already coated with Kodak-NTB-3 emulsion. After 5 weeks of exposure at low humidity (4°C) in a dark lead-lined cabinet, slides were developed at 17°C in Dektol for 2 min. Development was terminated by Kodak Liquid Hardener and slides were fixed in hypo (Kodak). After washing in running tapwater, sections were stained with pyronine Y, dried, mounted with Permount and viewed with a Zeiss Universal microscope. Control slides prepared for positive and negative chemography showed no significant fading of latent images or spurious generation of grains after 60 d of exposure. Grain counts are the means of 2,400 μm^2 areas.

Table 2 Regional localisation of the morphine-like factor and opiate receptors in bovine and rat brain

	Morphine-like factor (U per mg protein)	Opiate receptor binding (c.p.m. per 0.1 mg protein)
Bovine		
Caudate	480	320
Hypothalamus	250	282
Spinal cord	140	205
Pons	135	231
Cerebral cortex (Parietal)	80	173
Thalamus	75	179
Cerebellum	50	86
Medulla oblongata	50	88
Corpus callosum	10	61
Rat		
Caudate	480	900
Brainstem (midbrain)	140	220
Cerebral cortex	80	210
Cerebellum	None	None

Data are derived from the study of Pasternak and Snyder⁴¹. In assays for the morphine-like factor, brains were homogenised in ten volumes 0.32 M sucrose with a Potter-Elvehjem homogeniser, and centrifuged at 100,000g for 1 h. The pellet was resuspended in 2 volumes of 10 mM Tris-HCl buffer (pH 7.7 at 25 °C) immersed in a bath of boiling water for 15 min, recentrifuged at 100,000g for 1 h and the supernatant assayed by adding 50–200 µl to an opiate receptor binding assay with ³H-naloxone and 1 mM MnCl₂. Opiate receptor assays were performed as previously described²⁰. The experiments were replicated four times with less than 20% variation between experiments.

Mapping opiate receptor sites by autoradiography is still in a preliminary stage. It is however already clear that this information will help greatly in elucidating the physiological and pharmacological roles of opiate receptor sites. Neurophysiologists who would like to record from cells containing opiate receptor sites ought to focus on the locus coeruleus, the zona compacta of the substantia nigra and other areas enriched in these receptors.

The endogenous morphine-like factor

Man was not made with morphine in him. Is the opiate receptor 'designed' for a normally occurring morphine-like substance, perhaps a neurotransmitter? If so, such a system is widespread among animals. Opiate receptor binding with almost identical substrate specificity has been detected in the central nervous system of all vertebrates, and the most primitive fish have as many opiates receptors as mammals including man²⁷. Strikingly, no opiate receptor binding can be demonstrated in any invertebrates, which may indicate a relationship of the opiate receptor to known differences in patterns of vertebrate and invertebrate neuronal connections. The possibility that the opiate receptor is associated with a neurotransmitter system is strengthened by subcellular fractionation studies which indicate that in mammalian brain homogenates opiate receptor binding is most enriched in synaptosome fractions containing nerve endings with their associated postsynaptic membranes³⁸. When these are subjected to hypotonic lysis, opiate receptor binding is recovered primarily in the synaptic membrane fraction, as would be expected for a neurotransmitter receptor³⁸. A direct approach to identifying a possible neurotransmitter associated with the opiate receptor was adopted by Hughes³⁹, who found in brain extracts a substance which mimics the ability of morphine to inhibit electrically induced contractions of smooth muscle preparations such as the mouse vas deferens or guinea pig intestine, while Terenius⁴⁰ and Pasternak and Snyder⁴¹ reported that brain extracts compete for opiate receptor binding. The substance studied

by both approaches seems to be the same or very similar. The morphine-like factor or "enkephalin" is a peptide, degraded by carboxypeptidase A and B as well as leucine-aminopeptidase and to a lesser extent by chymotrypsin but not by trypsin^{39,41,42}. In opiate binding assays, the material behaves like an agonist, since its ability to compete for binding is impaired by sodium and protein modifying reagents but enhanced by manganese⁴¹. Its regional distribution in calf, rat and rabbit brain is closely similar to that of the opiate receptor itself (Table 2)^{39–42}. Preliminary chemical analysis indicates that the morphine-like factor contains three residues of glycine and one each of phenylalanine, tyrosine and methionine and may also contain tryptophan^{39,42}. A chemically different peptide, which also mimics the effect of morphine on smooth muscle, has been isolated from pituitary glands⁴³.

If the morphine-like factor is a neurotransmitter, how might it affect postsynaptic cells? Morphine and other opiates applied iontophoretically to single cerebral cortex cells inhibit firing in proportion to their pharmacological potency with marked stereospecific actions and antagonism by naloxone, suggesting that the morphine-like factor may be an inhibitory transmitter⁴⁴. Cells of rats addicted to morphine are no longer inhibited by administered morphine and may even show a paradoxical excitation. Because these changes with addiction occur at the opiate responsive cell itself, the locus of the fundamental alteration in opiate addiction would seem to be at the level of the cell membrane possessing the opiate receptor.

Possible mechanisms of addiction

The term "addiction" is not easily defined. Most people consider opiate addiction to comprise three major elements: tolerance, physical dependence, and compulsive craving. Tolerance means simply that, after prolonged administration of a drug, the organism can "tolerate" more of it, that is, it is now less sensitive to the drug than before. Physical dependence refers to the fact that when the drug is withdrawn or when the animal or person is treated with an opiate antagonist, withdrawal symptoms become evident which are usually in a direction opposite to the initial effects of the drug. Thus whereas morphine produces pupillary constriction, constipation and sedation, during withdrawal the pupils are dilated and there is diarrhoea and central excitation. Compulsive craving is very difficult to evaluate; it refers to the addict's propensity for the drug even long periods after he has been physically withdrawn. Just as tolerance can be looked on as a state of decreased sensitivity to opiate agonists, so in physical dependence animals and humans become more sensitive to antagonists. Withdrawal symptoms can be elicited by much lower doses of antagonists in severely than in mildly addicted animals.

There are two types of model which explain opiate addiction; one of them involves a change at the level of the opiate receptor or closely allied structures, whereas the other requires no such alterations^{45–48}. In the latter model, one presumes that morphine suppresses some neuronal system in the brain. To compensate, a completely separate system increases its activity to counteract the suppressive action of morphine; whereupon the organism is "tolerant". When morphine is withdrawn, the "overactive" second system produces withdrawal symptoms.

Hypersensitivity to antagonists coupled with subsensitivity to agonists in addiction is reminiscent of the properties of the sodium or "antagonist" state of the opiate receptor as contrasted to the no-sodium or "agonist" state. It may be speculated that tolerance and physical dependence reflect a change in the opiate receptor so that it is less capable of assuming the agonist form, favouring instead the antagonist, sodium form. If this were so, addicted animals should be "subsensitive" to opiate agonists, since the receptor would

be less frequently in the "agonist" state to mediate drug effects. Similarly, the organism would be supersensitive to antagonists because of the predominant sodium or antagonist state of the receptor. Though opiate receptor assays both *in vitro* and *in vivo* have failed to show systematic changes related to the addicted state^{13,50}, it is conceivable that such a subtle alteration may be reflected only in membrane properties which occur secondarily to the binding itself. In this connection, recent studies of opiate effects on a neuroblastoma-glioma hybrid in cell culture are illuminating. Opiate agonists, in proportion to their affinity for opiate receptor binding sites in this clone, decrease adenylate cyclase^{51,52} and enhance the accumulation of cyclic GMP⁵³. Opiates also reverse the stimulation of adenylate cyclase by prostaglandin E₁ and adenine⁵¹. Cells exposed chronically to morphine become "tolerant" to the effects of morphine so that previously active doses no longer antagonise the prostaglandin stimulation of adenylate cyclase⁵⁴. When the cells are put into "withdrawal" by naloxone, they become "supersensitive" to the ability of prostaglandin E₁ to stimulate adenylate cyclase, an effect opposite to that of morphine itself in drug-naïve cells⁵⁴.

Of course, one might argue that the cyclic nucleotide changes are relevant only to these cancerous cells of the nervous system in tissue culture. Previously, Collier and Roy⁵⁵ had described a prostaglandin-stimulated adenylate cyclase in mammalian brain which is inhibited by morphine and other opiates, and may be related to the neuroblastoma-glioma effects. Moreover phosphodiesterase inhibitors, which elevate brain cyclic AMP levels, elicit in rats behavioural changes resembling the opiate withdrawal syndrome and which are enhanced by as little as 0.03 mg kg⁻¹ of naloxone^{56,57}.

In summary, recent identification of opiate receptor sites in the central nervous system by biochemical means has spurred a great body of research directed both at pharmacological actions of opiates as well as at the normal functioning of the opiate receptor. An apparently novel peptide neurotransmitter, the morphine-like factor, is being characterised.

As in the case with numerous hormones and neurotransmitters, this morphine-like factor may have as its second messenger cyclic nucleotides, either cyclic AMP or cyclic GMP. Most dramatically, it seems possible that changes in the adenylate cyclase associated with the opiate receptor and conceivably in the opiate receptor itself may begin to answer the many riddles of addiction.

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articles

Quark charges and nuclear β -decay

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The rate of β -decay is affected by the charges, Q , of the nucleon's constituent quarks. The Salam-Weinberg scheme permits us, in a spirit of adventurous naivety, to conclude that $\bar{Q} = 0.20 \pm 0.10$; this combines with electron and neutrino data to suggest individual quark charges: $Q_p = 0.69 \pm 0.07$, $Q_n = -0.29 \pm 0.15$.

The quark-parton model

The highly successful quark model for the description of hadronic spectra^{1,2} considers baryons as made up of three quarks of baryon number 1/3 and of charges that, in the classical version of the model, are $Q_t = 2/3$ and $Q_n = -1/3$ times the electronic unit but that, in alternative versions, may be integral. The parton model of the nucleon, originally inspired by the scaling

phenomena of deep inelastic electron scattering and by high energy hadronic phenomena^{3,4}, considers the nucleon to be made of "point-like" subunits—the partons. Identification of quarks with partons gives the quark-parton model.

The literal parton model is encouraged by several phenomena such as:

- (1) the surprisingly large abundance of particles produced with high transverse momentum in pp collisions at high energy such as the 28+28 GeV experiments at the CERN ISR⁵;
- (2) the linearity of neutrino-nucleon total cross section with neutrino energy up to $E \simeq 200$ GeV (ref. 6); the latter data indicate that the total effective dimension of the νN interaction, including that of the parton, is less than 0.02 fm which, on comparison with $\langle r^2 \rangle^{1/2} \simeq 0.8$ fm for the nucleon as a whole, suggests that the partons are, at the most, 2–3% of the size of the nucleon that they make up.

That the spin of the partons is $\frac{1}{2}$ follows experimentally from the deep inelastic electron scattering experiments (the Callen-Gross relation)⁶; the experimental ratio of 3:1 between high energy neutrino and antineutrino cross sections on nucleons then shows that the nucleon is composed 95% or more of partons and therefore has very little antiparton content⁶ (some antimatter there must be since forces must make pairs). The Gross-Llewellyn-Smith sum rule for neutrino-nucleon interactions permits the "counting" of the partons; the result of 3.2 ± 0.6 completes the "experimental" identification of the quark-parton model⁶.

Of course, one is not obliged to talk this language at all. One can replace the literal constituent quarks by current quarks and one can replace the literal point-like partons by statements about singularities of relevant commutators on the lightcone. But the quark-parton model provides, at least, a legitimate, and attractively picturesque, framework for the parameterisation of particle structure and, within that framework, it may be asked whether an internal consistency with one or other version of the model is found. In this article I adopt the simple quark-parton viewpoint, ignore its naiveties and illuminate it through ordinary low energy nuclear β -decay.

The essential point is that β -decay does not involve solely the weak interaction; electromagnetic forces must be simultaneously at work providing the "radiative corrections". The radiative corrections involve not only the nucleon as a whole but also its constituent quarks so the magnitude of the radiative corrections must involve the charges of the quarks; this article makes statements about those charges.

We proceed through a hierarchy of hypotheses: (1) conservation of the vector current⁷; (2) μ -e universality in the Cabibbo sense⁸; (3) unification of the weak and electromagnetic interactions in the Salam-Weinberg sense⁹. Without (1) and (2) the present remarks fall to the ground. Adoption of (3) makes our evaluation specific but it could be redone for any other version of the gauge theories than Salam-Weinberg and any other version of the quark model than those used by Sirlin¹⁰ in his contingent treatment of the radiative corrections. In view of the uncertainties attaching to (3) the present analysis should perhaps be regarded as no more than illustrative of the sort of information as to the quark charges that will ultimately be extracted from nuclear β -decay; but if, as seems possible, Sirlin's result for the radiative corrections has a rather general validity, we may already regard the present outcome as of interest. We find:

$$\bar{Q} = 0.20 \pm 0.10$$

where $\bar{Q}$ is the mean quark charge of the nucleon (compare 1/6 expected for the classical non-integrally-charged quark model^{1,2} and 1/2 for integrally-charged quark models).

The radiative corrections

Briefly, it is clear^{11,12} that the radiative corrections of order α to nucleon β -decay may be divided into two parts: the well-understood explicit "outer" correction δ_p^a is energy-release dependent but independent of nucleon structure and β -decay anatomy

and may therefore be absorbed into the phase-space factor; the "inner" correction Δ_{β^a} depends on structure and anatomy but is energy-release independent so may be absorbed into the coupling constant. We particularise to vector β -decay and write:

$$g_{\beta^a}^V = g_{\beta^a}^V (1 + \frac{1}{2} \Delta_{\beta^a}^V)$$

where $g_{\beta^a}^V$ is the vector coupling constant as we should know it but for the radiative corrections.

Our hope of quark-charge information therefore resides in $\Delta_{\beta^a}^V$. The trouble about $\Delta_{\beta^a}^V$ used to be the divergencies that it contained: these were severe and largely intractable, even when the primitive point 4-fermion interaction $N \rightarrow N e \nu$ (ref. 13) was replaced by mediation through the hypothetical intermediate vector boson $N \rightarrow N W; W \rightarrow e \nu$ (ref. 14) and when quark structure was introduced¹⁵. The new unified weak/electromagnetic gauge theories⁹ have, however, changed all that: $\Delta_{\beta^a}^V$ is now divergenceless apart from a single term that can, unexceptionably, be absorbed into the weak coupling constant as a universal renormalisation in direct analogy to the hallowed practice in quantum electrodynamics.

$\Delta_{\beta^a}^V$ must be evaluated in the context of specific quark models and specific formulations of the unified theory; so far only two classes of quark model have been studied in detail¹⁰ and one formulation of weak interaction theory, namely the Salam-Weinberg scheme⁹; however, the general form of $\Delta_{\beta^a}^V$ has remained remarkably stable against changes in its conceptual background^{10,13-15} and it seems probable that something very like the present form will remain no matter what the quark and weak interaction pictures maybe come. We therefore conduct our present analysis using Sirlin's¹⁰ evaluation of $\Delta_{\beta^a}^V$, recognising that the Salam-Weinberg scheme on which it is based is by no means proven and recognising the limited range of quark pictures for which it has been shown to be valid but believing that something very like it will continue to hold and therefore regarding the conclusions that we tentatively draw here as being, at the least, illustrative of the quality of information that can, in principle, be extracted from β -decay data.

We can choose whether we want to use β -decay to tell us about nucleon structure or about the anatomy of β -decay. The earlier viewpoint, pioneered by Blin-Stoyle, to whom a great deal is due in studies of the present type¹⁶, was that β -decay suggested mediation of the weak interaction by a very heavy intermediate vector boson; the success of the gauge theories now seems to recommend that we tentatively take the weak interaction for granted and use β -decay to tell us about nucleon structure; this is the viewpoint of my present note.

What is of interest is not so much $\Delta_{\beta^a}^V$ itself as $\Delta_{\beta^a}^V - \Delta_{\mu^a}$ where Δ_{μ^a} is the analogous quantity involved in muon decay. Sirlin¹⁰ finds

$$\Delta_{\beta^a}^V - \Delta_{\mu^a} = (\alpha/2\pi) \{ 3 \ln(m_Z/m_N) + 6 \bar{Q} \ln(m_Z/m_A) \}$$

The first term in curly brackets comes from the vector part of the hadronic weak current; the second term comes from the axial part of the hadronic weak current which contributes to the overall vector amplitude because it can combine appropriately with the isoscalar part of the electromagnetic current. m_N is the nucleon mass and m_Z that of the neutral intermediate vector boson, Z^0 , of the Salam-Weinberg scheme (the mass of the charged $W^\pm$ drops out in $\Delta_{\beta^a}^V - \Delta_{\mu^a}$). m_A is the mass of some suitable "axial" structure and is conventionally awarded the mass of the A_1 -meson for which we take 1,300 MeV (ref. 17 and M. G. Bowler, private communication); this is the energy above which the asymptotic form for the axial current part of the second rank tensor that describes the exchange of virtual photons between nucleon and decay electron becomes valid. m_A is uncertain but it would have to be raised to 4.5 GeV to shift our present conclusion as to $\bar{Q}$ by its error limit.

When we are concerned with nuclear, as opposed to nucleon,

β -decay "outer" radiative corrections arise in addition to δ_β^a . If one writes them as $\delta_\beta^{za^m}$ a general restriction is $m \geq n$ (ref. 18). By the Ademollo-Gatto theorem¹⁹, which says that the renormalisation of coupling constants is of second order in the multiplet mass splittings, there is no term linear in $Z\alpha$ (ref. 18). Higher terms with $m = n$ and not involving nuclear excitation are negligible²⁰; those involving nuclear excitation are treated

such as has been occasionally suggested as an explanation for the anomalously high rate of $\eta \rightarrow \pi$ decay, would have negligible effect²³).

For such $J\pi = 0^+$, $T = 1$ decays, including all radiative corrections, we can therefore write:

$$f^R t = \frac{\pi^3 \hbar^7 \ln 2}{m^6 c^4 g_{\beta V}^R z^2 (1 - \epsilon)} = \frac{6.153 \times 10^{-95}}{g_{\beta V}^R z^2 (1 - \epsilon)} \text{ erg}^2 \text{ cm}^6 \text{ s}$$

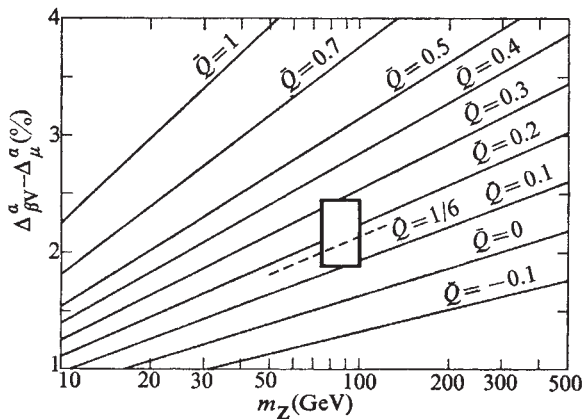


Fig. 1

separately as small modifications to the nuclear matrix element as I shall discuss in detail. Of the terms with $m = n+1$, $\delta_\beta^{za^2}$ and $\delta_\beta^{za^3}$ have been evaluated^{21,22} and the remainder shown to be negligible (for the nuclei of present concern). Terms with $m = n+2$ and so on are also negligible^{21,22}; we therefore, with an overall accuracy of better than 0.1%, replace the ordinary phase space factor f (which already contains the effect of the gross Coulomb field of the nucleus in distorting the outgoing electron wave) by its outer-radiative-corrected version f^R :

$$f^R = f(1 + \delta_\beta^a + \delta_\beta^{za^2} + \delta_\beta^{za^3})$$

(The δ_β terms are designed to be combined in this way)

We remember that other radiative effects are contained in $g_{\beta V}^R$ and in the modification of the nuclear matrix element.

Pure vector nuclear β -decay

β -decay within isospin multiplets of $J\pi = 0^+$ is pure vector because there is no nuclear spin to flip. Under complete charge independence the matrix element would be given purely by the geometrical Clebsch-Gordan factor in the isospins and, for the cases of $T = 1$ with which we are here concerned, would take the value $\sqrt{2}$. In practice, charge independence is relaxed by the implicit and explicit effects of the Coulomb force so that the matrix element must be multiplied by $(1 - \epsilon)^{1/2}$ where we expect, by the Ademollo-Gatto theorem, that ϵ should go roughly as Z^2 . (Non-Coulomb breaking of charge independence,

where t is the half life (corrected for branching and orbital electron capture) and where there is no error in the numerical value to the accuracy stated.

f itself must be computed with care and must involve solving the Dirac equation in a field generated by a nuclear charge distribution of finite size, consistent with electron scattering and muonic X-ray data, screened by the atomic electrons, and convoluting the resultant positron and associated neutrino wavefunctions with nucleonic wavefunctions generated in a realistic nuclear potential. This task has been performed with an overall reliability of better than 0.05% (refs 24 and 25).

The ϵ have been computed several times recently and their associated error is probably better than 0.1%; this matrix element correction can also be derived empirically from the experimental data (see section under that heading), as reviewed in the section headed ϵ -values, when it shows good agreement with the directly-computed values.

The experimental data relate to the decay of ^{14}O , $^{26}\text{Al}^m$, ^{34}Cl , $^{38}\text{K}^m$, ^{42}Sc , ^{46}V , ^{50}Mn and ^{54}Co for each of which bodies $f t$ is determined to better than $\pm 1/3\%$. The set shows excellent internal consistency and we conclude

$$g_{\beta V}^R = (1.4116 \pm 0.0008) \times 10^{-49} \text{ erg cm}^3$$

Conserved vector current and the Cabibbo angle

The excellent internal consistency of the eight accurately-measured vector transitions agrees with the hypothesis of the conserved vector current⁷ in that vector β -decay suffers no renormalisation by mesonic exchange effects and may therefore be equated with muon decay if we extend the hypothesis to a universality of the weak coupling constant in analogy to that of electric charge. In adopting the hypothesis of universality we must follow Cabibbo⁸ and divide the hadronic weak current into strangeness-conserving and strangeness-changing parts so that our expectation becomes:

$$g_{\beta V} = g_\mu \cos \theta_C$$

where θ_C is the Cabibbo angle that splits the hadronic decay strength between $\Delta S = 0$ and $\Delta S = 1$.

This expectation is not vitiated by W-boson propagator corrections which are of order $(m_\mu/m_W)^2$ or less, that is less than 0.01% for $m_W > 10 \text{ GeV}$ as experimentally demonstrated⁶, nor by finite nucleon size effects which amount to $(2/21)$

Table 1

Decaying body	$E_0(\text{keV})$	$t(\text{s})$	$f^R t(\text{s})$	$f t \text{ s}$
^{14}O	$1,809.86 \pm 0.36$ (ref. 24)	71.136 ± 0.031 (ref. 24)*	$3,094.3 \pm 3.0$	$3,090.3 \pm 4.3$
$^{26}\text{Al}^m$	$3,210.7 \pm 0.5$ (ref. 36)	6.352 ± 0.005 (ref. 24)	$3,085.9 \pm 3.3$	$3,080.3 \pm 4.5$
^{34}Cl	$4,467.9 \pm 1.3$ (ref. 36)	1.531 ± 0.004 (ref. 36)	$3,100.7 \pm 9.0$	$3,088.0 \pm 9.5$
$^{38}\text{K}^m$	$5,021.2 \pm 3.4$ (ref. 37)	0.9264 ± 0.0007 (ref. 37)	$3,103.4 \pm 9.9$	$3,091.9 \pm 10.4$
^{42}Sc	$5,399.9 \pm 2.2$ (ref. 36)	0.6846 ± 0.0009 (ref. 24)	$3,104.7 \pm 7.1$	$3,092.3 \pm 7.8$
^{46}V	$6,018.8 \pm 2.8$ (ref. 36)	0.4258 ± 0.0007 (ref. 38)	$3,099.1 \pm 8.4$	$3,090.1 \pm 9.0$
^{50}Mn	$6,607.8 \pm 2.1$ (ref. 36)	0.2837 ± 0.0006 (refs 38, 39)	$3,097.0 \pm 8.0$	$3,086.8 \pm 8.6$
^{54}Co	$7,219.0 \pm 1.6$ (refs 36, 40)	0.1934 ± 0.0003 (refs 38, 40)	$3,102.9 \pm 5.3$	$3,090.8 \pm 6.1$

* And J. A. Becker, R. L. Chalmers, B. A. Watson, and D. H. Wilkinson, unpublished.

$W_0^2 \langle r^2 \rangle_{\beta\gamma}$, where W_0 is the energy release in natural units, 0.01% in the worst of our present cases.

The Cabibbo angle is most naturally determined from semi-leptonic hyperon decay on which a large and excellently self-consistent body of data exists²⁶ and which, after a small allowance for SU(3) symmetry breaking²⁷, yields $\sin\theta_c = 0.238 \pm 0.005$. Another approach to θ_c comes from K_{e3} -decay which, interpreted conventionally using the Klein-Gordon equation for the pion, yields²⁸ $\sin\theta_c = 0.223 \pm 0.003$. Since K-decay is not so closely connected to the nucleon-decay problem as is hyperon decay, since it has fewer internal consistency checks and since there is some doubt about the appropriate treatment of the pion²⁸, we do not try to combine the two values of $\sin\theta_c$ but rather accept the K_{e3} value as evidence that the hyperon value may be a bit on the high side and so reduce the latter by its own standard deviation and adopt

$$\sin\theta_c = 0.233 \pm 0.005$$

Muon decay

The mean-life of the muon, τ_μ , is given by

$$\tau_\mu = \frac{192\pi^3 \hbar^7 (1 - \delta_\mu^\alpha)}{m_\mu^5 c^4 (1 - 8m_e^2/m_\mu^2) g_\mu^R{}^2}$$

where δ_μ^α is the "outer" radiative correction of order α , the analogue of δ_β^α , and is given by^{29,30}

$$\delta_\mu^\alpha = -(\alpha/2\pi)(\pi^2 - (25/4))$$

$\delta_\mu^{\alpha^2}$, and higher corrections have been evaluated only incompletely³¹ but seem unlikely to introduce changes of more than 1 part in 10^4 in τ_μ . g_μ^R contains the effect on τ_μ of the anatomy of the β -decay process

$$g_\mu^R = g_\mu(1 + \frac{1}{2}\Delta_\mu^\alpha)$$

Recent experiments give

$$\begin{aligned}\tau_\mu &= 2197.3 \pm 0.3 \text{ ns} \quad (\text{ref. 32}) \\ &= 2197.11 \pm 0.08 \text{ ns} \quad (\text{ref. 33})\end{aligned}$$

so that

$$g_\mu^R = (1.4358 \pm 0.0001) \times 10^{-49} \text{ erg cm}^3$$

where the error has been inflated to take account of the uncertainties in $\delta_\mu^{\alpha^2}$.

$$\Delta_{\beta\gamma}^\alpha - \Delta_\mu^\alpha$$

We bring together the various values quoted above to find

$$\Delta_{\beta\gamma}^\alpha - \Delta_\mu^\alpha = 2 \left\{ \frac{g_{\beta\gamma}^R}{g_\mu^R \cos\theta_c} - 1 \right\} = (2.19 \pm 0.27)\%$$

which we could confront, to find $\bar{Q}$, with Sirlin's above-quoted theoretical expression for $\Delta_{\beta\gamma}^\alpha - \Delta_\mu^\alpha$, if we knew m_Z .

M_Z

Within the Salam-Weinberg scheme, that we have adopted, the mass, m_Z , of the neutral intermediate vector boson that figures in Sirlin's formula for $\Delta_{\beta\gamma}^\alpha - \Delta_\mu^\alpha$ is given by: $m_Z = 74.6/\sin(2\theta_w)$ GeV; θ_w is the Weinberg angle that relates the weak and electromagnetic coupling constants. The scheme is encouraged by the "neutral current" neutrino interactions that for muon-less hadron-producing events, within a quark-parton model such as we consider here, give $\sin^2\theta_w = 0.39 \pm 0.05$ (ref. 34). $\bar{\nu}_e e$ -scattering (to which charged currents can also contribute) gives $\sin^2\theta_w < 0.3$ (ref. 35). $\bar{\nu}_\mu e$ -scattering is not

inconsistent with $\sin^2\theta_w \simeq 0.25$ (ref. 34 and D. H. Perkins, private communication), somewhat smaller and somewhat larger values being admissible. For our present purpose we therefore use:

$$74.6 < m_Z < 100 \text{ GeV}$$

where 100 GeV corresponds to $\sin^2\theta_w \simeq 0.17$ which appears to be safely on the low side.

$\bar{Q}$

Our experimental $\Delta_{\beta\gamma}^\alpha - \Delta_\mu^\alpha$ and the above-adopted m_Z are confronted with Sirlin's formula in Fig. 1; we derive: $\bar{Q} = 0.20 \pm 0.10$. (The largest component in the error comes from that in θ_c .)

We repeat the various cautionary remarks about our reliance on the hypotheses listed above, in particular on the Salam-Weinberg scheme, and on Sirlin's derivation of his formula, but within these limits our value of $\bar{Q}$ can be treated as a definite parameter.

Quark charges

$\bar{Q} = 0.20 \pm 0.10$ is consistent with $\bar{Q} = 1/6$ expected for the classical quark model; it is inconsistent with $\bar{Q} = 1/2$ as for integral quarks. Other data relevant to "quark charges" come from a comparison of deep inelastic neutrino and electron scattering; the two show good internal consistency within the quark-parton model and their comparison then permits the statement, if there are no strange constituents to the nucleon, which is a good assumption in view of the evident lack of anti-matter remarked on above: $\bar{Q}^2 = 0.28 \pm 0.03$ (ref. 6) which is consistent with the expectation of the classical quark model: $\bar{Q}^2 = 5/18$.

Putting together $\bar{Q}$ and $\bar{Q}^2$, the "experimental" values of the separate quark charges become

$$\begin{aligned}Q_p &= 0.69 \pm 0.07 \\ Q_n &= -0.29 \pm 0.15\end{aligned}$$

to be compared with the classical values: $Q_p = 2/3$, $Q_n = -1/3$.

It is the especial virtue of the present approach that it is sensitive to $\bar{Q}$ rather than to $\bar{Q}^2$.

Experimental data

J. M. Freeman, W. E. Burcham and their colleagues at AERE, Harwell have, for many years, carried out accurate measurements on super-allowed Fermi β -decay. More recently, important contributions have also come from D. E. Alburger, J. C. Hardy and their colleagues at Brookhaven National Laboratory and Chalk River. Old inconsistencies have now disappeared; the present situation, summarised in Table 1, where t has been corrected for branching and electron capture, is one in which considerable confidence may be resposed.

E_0 is the maximum kinetic energy of the positron. $\mathcal{F}$ is defined as $f^R(1-\epsilon)$. The ϵ -values have been taken from Table 2 and an error of $\pm 0.1\%$ associated with each. $\mathcal{F}t$ should then be a constant if the vector current is conserved; its mean value

Table 2

A	$\epsilon_1\%$ (ref. 43)	$\epsilon_1\%$ (ref. 44)*	$\epsilon_1\%$ (ref. 24)	$\epsilon_2\%$ (ref. 24)	$\epsilon\%$
14	0.04	0.04	0.15	0.05	0.13
26	0.10	0.11	0.12	0.07	0.18
34	0.17	0.18	0.19	0.23	0.41
38	0.21	0.21	0.21	0.16	0.37
42	0.25	0.25	0.31	0.13	0.40
46	0.30	0.29	0.17	0.04	0.29
50	0.35	0.33	0.21	0.03	0.33
54	0.40	0.38	0.27	0.04	0.39

* And A. M. Lane, private communication.

is $\bar{f}t = 3,087.7 \pm 2.3$ s with $\chi^2/\nu = 0.56$ so internal consistency is good.

Alternatively, we may follow the suggestion of the Ademollo-Gatto theorem¹⁹ namely that ϵ should go, *ceteris paribus*, as the square of the mass-splittings within the isomultiplets which, over the range of Table 1, means roughly as $Z^{1.75}$. Fitting to the form: $f^R t = (f^R t)_{Z=0} + aZ^{1.75}$, gives $(f^R t)_{Z=0} = 3,088.5 \pm 3.8$ s in good agreement with $\bar{f}t$. We adopt $3,087.9 \pm 3.5$ s; this leads to the $g_{\beta\gamma}^R$ -value quoted in the text.

The ϵ -values

There is a long history to computation of the ϵ -values⁴¹. Recent estimates are shown in Table 2. ϵ splits into two parts: ϵ_1 is due to the one-body Coulomb force and ϵ_2 to the two-body Coulomb force. The three estimates of ϵ_1 , made by very different methods, agree well among themselves (the third of them has been halved to allow more properly for the parentage spectrum⁴³, except for ⁴²Sc where the parentage is unique). It seems that $\epsilon = \epsilon_1 + \epsilon_2$ as quoted should be reliable to $\pm 0.1\%$.

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In vitro synthesis of tRNA precursors and their conversion to mature size tRNA

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Two *Escherichia coli* tRNA gene clusters, $tRNA_1^{Tyr}$ (su_3^+ and su_3^-) and $tRNA_2^{Tyr}$, $tRNA_2^{Gly}$ (su_3^+), $tRNA_3^{Thr}$, were transcribed in a purified *in vitro* system. Evidence indicates that the adjacent tRNA genes are transcribed together as a common precursor of large size, which, on incubation with crude cell extracts, yields mature tRNA molecules.

It is now generally established that tRNA genes of bacterial and mammalian cells as well as those of bacteriophage T4 are transcribed into large RNA precursor molecules which are subsequently cleaved by specific nucleases to yield mature size tRNA^{1–6}. Because of the transient nature of the tRNA precursors, however, only in a few cases have such molecules been isolated *in vivo*^{1,7} and some of them may represent already partially cleaved precursors^{8,9}. Transcription of tRNA genes *in vitro* by purified RNA polymerase has the advantage of producing completely unmodified tRNA precursors and provides an opportunity to study in detail the processing and modification of primary transcription products.

The structural genes for some tRNA molecules are often present on the *E. coli* chromosome as clusters containing identical genes^{10,11}, or genes specifying different tRNAs¹². Two such tRNA gene clusters are being studied: the duplicate genes coding for $tRNA_1^{Tyr}$ (su_3^+ and su_3^-) carried by transducing phage $\phi 80psu_3^{+-}$ (ref. 13) and the $tRNA_2^{Gly}$ (su_3^+) $tRNA_3^{Thr}$, $tRNA_2^{Tyr}$ genes carried by $\lambda h80T$ phage DNA¹². *In vivo*^{8,14} and *in vitro*^{15,16} studies suggest that these tRNA genes may be transcribed as large RNA precursors. In the

present report the multiple tRNA genes carried by $\phi 80psu_3^{+-}$ and $\lambda h80T$ phage DNA were transcribed *in vitro* by *E. coli* RNA polymerase as polycistronic tRNA precursors. These precursors were cleaved on incubation with a supernatant fraction prepared from *E. coli*, yielding mature size tRNA molecules. The process of precursor cleavage was analysed using polyacrylamide gel electrophoresis. It was found that the formation of tRNA takes place by way of intermediate size RNA molecules. The mature size tRNA and some of the intermediate precursors were found, using fingerprint techniques, to be similar or identical to those made *in vivo*.

Transcription and processing of $tRNA_1^{Tyr}$ precursor

$\phi 80psu_3^{+-}$ DNA carrying both suppressor and wild-type $tRNA_1^{Tyr}$ genes was transcribed *in vitro* by RNA polymerase in a reaction mixture containing α -³²P-GTP. After 30 min incubation at 37 °C, RNA synthesis was terminated by the addition of actinomycin D and DNase 1. The formation of mature size $tRNA_1^{Tyr}$ from larger precursors was studied by following the processing of the *in vitro* $\phi 80psu_3^+$ RNA transcripts by crude *E. coli* S100 extracts as a function of incubation time.

The synthesised α -³²P-GMP-labelled RNA was extracted from a portion of the reaction mixture (zero time of processing). The rest of the reaction mixture, was incubated with S100 extract from *E. coli*. At different time intervals (5, 10, 20 and 40 min) equal aliquots were removed, the RNA extracted by phenol and precipitated by ethanol. The RNA preparations were separated by electrophoresis on a 5% polyacrylamide-7 M

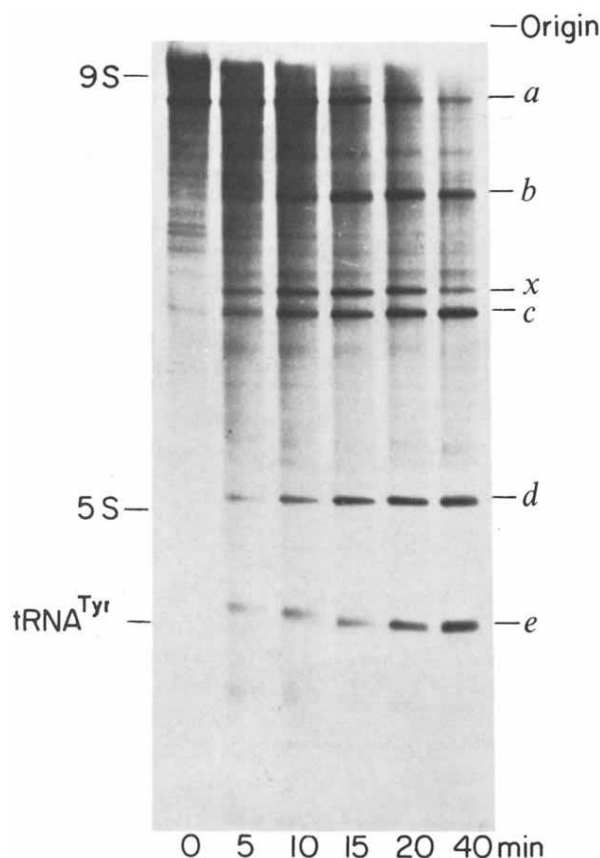


Fig. 1 Autoradiogram of acrylamide gel separations of cleavage products of RNA transcribed *in vitro* on $\phi 80psu_3^{+/-}$ DNA. $\phi 80psu_3^{+/-}$ DNA (20 μ g) was transcribed in 200 μ l reaction mixture containing: 0.05 M Tris-HCl (pH 7.9), 0.01 M $MgCl_2$, 0.075 M KCl, 1 mM dithiothreitol, 0.4 mM each of ATP, CTP and UTP, 0.3 mM α - ^{32}P -GTP (2.3 Ci mmol $^{-1}$; Amersham, England) 12 μ g ρ termination factor and 60 U RNA polymerase (1,600 U mg $^{-1}$). The synthesis was initiated after 5 min pre-incubation at 38 $^{\circ}C$ by addition of nucleoside triphosphates. After 30 min incubation at 38 $^{\circ}C$ the reaction was stopped by the addition of 20 μ g actinomycin D and 4 μ g DNase I (Worthington, Freehold, New Jersey). RNA was extracted from a 30 μ l aliquot of the reaction mixture and run on acrylamide gel to give the electrophoretic pattern for zero time of processing. The rest of the mixture was incubated at 37 $^{\circ}C$ with 400 μ g S100 extract. Aliquots were removed at the times indicated in the figure and fractionated by acrylamide gel electrophoresis on 40 $\times$ 20 cm 5% gels (20:1 acrylamide:bis-acrylamide) containing 7 M urea, in Tris-borate buffer¹⁷ at 175 V for 16–19 h. 9S globin mRNA, 5S RNA and tRNA^{Tyr} were run separately as reference markers and were located by staining the gel with Stains all (Eastman-Kodak). DNA-dependent RNA polymerase was prepared from *E. coli* MRE600 cells¹⁸ and further purified by low salt glycerol gradient centrifugation¹⁹. Termination factor ρ was prepared from *E. coli* MRE600 cells as described by Roberts²⁰. S100 supernatant fraction was prepared from log-phase *E. coli* MRE600 cells suspended in buffer (0.01 M Tris-acetate, pH 7.5, 0.014 M $MgCl_2$, 0.06 M KCl, 6 mM 2-mercaptoethanol) and disrupted in a French press. The extract was centrifuged for 90 min at 105,000g in a Spinco ultracentrifuge and the S100 supernatant obtained was dialysed against the same buffer and stored at $-20^{\circ}C$.

urea gel. An autoradiogram of the gel separation for the RNA preparations is presented in Fig. 1. A single RNA band is observed in the non-processed $\phi 80psu_3^{+/-}$ RNA (band *a* at 0 time of incubation). On incubation with the S100 extract, the intensity of this band decreases. Band *e*, with the mobility of tRNA^{Tyr}, appears only after addition of the S100 extract and increases in intensity with the time of incubation. Attention should be drawn to the fact that band *e* RNA reaches the size of mature tRNA^{Tyr} only after 15–20 min incubation. The varied

dispersity and larger size of band *e* RNA at shorter periods of incubation may be due to insufficient trimming of the additional nucleotide sequences by an exonuclease activity present in the S100 extract. Figure 1 also shows that the maturation of tRNA^{Tyr} is a stepwise process involving the formation of intermediate size RNA molecules. Concomitant with the disappearance of band *a* during incubation with the S100 extract, additional RNA bands *b*, *c*, *d* and *x* are formed, the intermediate character of which is suggested by the fact that during incubation their intensity increases, reaches a maximum and decreases again. This behaviour is clearly seen in the case of band *x* for the incubation periods covered by Fig. 1. For sufficiently long incubation periods all intermediate RNA bands virtually disappear (data not shown).

Transcription and processing of tRNA^{Tyr}, tRNA^{Gly}, tRNA^{Thr} precursor

λ h80T DNA was transcribed *in vitro* by RNA polymerase in a reaction mixture containing α - ^{32}P -GTP. The formation of tRNA molecules from precursors was studied by following the cleavage of the *in vitro* λ h80T RNA by *E. coli* S100 extracts as a function of incubation time. Figure 2 shows that the primary transcription product of λ h80T DNA (0 time of incubation with S100 extract) is composed of RNA molecules of varied size, which remain at the origin or migrate in the 9S region of the gel, and of three distinct bands *a*, *c* and *g*. On incubation with S100 extract, two 4S RNA bands (*e* and *f*) appear, their intensity increasing with time. Band *e* has a mobility corresponding to a tRNA^{Tyr} molecule. Band *f*, as shown later, is actually composed of two tRNA species, tRNA^{Thr} and tRNA^{Gly}, which do not separate on the urea-containing polyacrylamide gel. As observed previously for the formation of tRNA^{Tyr} from the $\phi 80psu_3^{+/-}$ DNA transcript (Fig. 1), the tRNA species in bands *e* and *f* (Fig. 2) appear first as diffuse bands and reach the mature tRNA size only after 15 min incubation. The

Fig. 2 Autoradiogram of acrylamide gel separation of cleavage products of RNA transcribed *in vitro* on λ h80T DNA. RNA synthesis and cleavage by S100 extract was as described in Fig. 1.

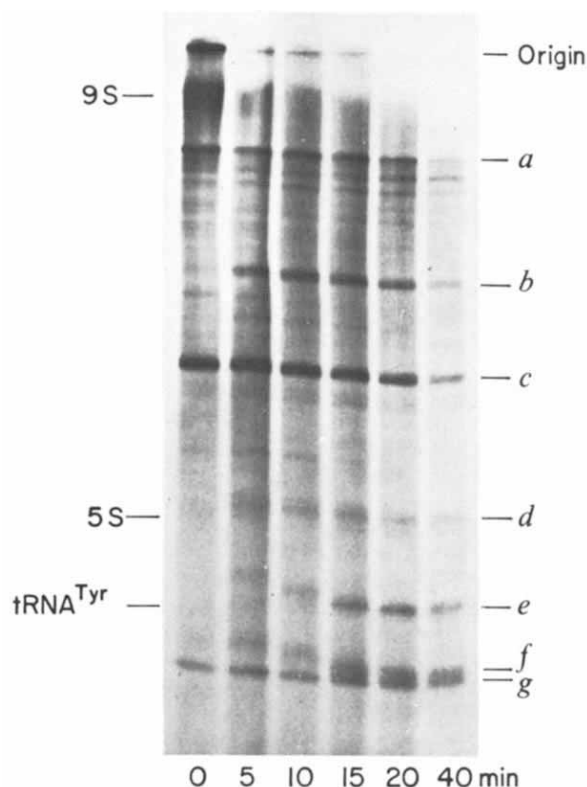


Table 1 Nearest-neighbour analysis of T_1 RNase oligonucleotides from *in vitro* tRNA $_1^{Tyr}$ and tRNA $_2^{Tyr}$

Spot no.	Corresponding tRNA $_1^{Tyr}$ and tRNA $_2^{Tyr}$ sequence	Nearest-neighbour predicted	Nearest-neighbour found
1	G!	G	G
2	G	G	G
3	CG(G)	C,G	C,G
4	CCG	C	C
5	AG	A	A
6	CAG	A	A
7	AAG(G)	A,G	A,G
8	CCAAG(G)	A,G	A,G
9	UG(G)	U,G	U,G
10	pGp	G	G
11	UG!(G)	U,G	U,G
12	pGp!	G	G
13	—	—	U
14	TψCG	C	C
15	UUCGG	C	C
16	UCAUCG*	C	C
17	ACUUCG	C	C
18	UAAAψCUG	U	U
19	ACUCUAAAψCUG*	U	U
20	ACUG	U	U
21	UCACAG†	A	A

Oligonucleotides were eluted from the two-dimensional fingerprints and hydrolysed with 0.3 M KOH at 37 °C. Resulting nucleotides were separated by paper electrophoresis at pH 3.5 and identified by autoradiography.

* Fragment derived from tRNA $_1^{Tyr}$ (ref. 22).

† Fragment derived from tRNA $_2^{Tyr}$ (ref. 22).

appearance of the tRNA bands is accompanied by the cleavage of band *a* (Fig. 2). For longer periods of incubation the disappearance of the other bands was also observed (data not shown).

Sequence analysis of tRNA molecules synthesised *in vitro*

The synthesised tRNA molecules were identified by the two-dimensional fingerprint of T_1 RNase digestion fragments²¹. As *in vitro* transcription was carried out with α -³²P-GTP and T_1 RNase cleaves the RNA adjacent to GMP residues, each fragment obtained contains a labelled 3'-terminal GMP. The two-dimensional fingerprint of the tRNA synthesised *in vitro* should therefore be the same as that obtained with the respective uniformly labelled *in vivo* tRNA species. The only exception is the 3'-terminal fragment of the tRNA which contains no GMP and will not be labelled in the *in vitro* transcript. Autoradiograms of the two-dimensional T_1 RNase fingerprints of band *e* (tRNA $_1^{Tyr}$) from Fig. 1 and band *e* (tRNA $_2^{Tyr}$) from Fig. 2 are presented in Fig. 3*a* and *b*, respectively. The two fingerprints are similar to each other and to the T_1 RNase fingerprint of *in vivo* *E. coli* tRNA $_1^{Tyr}$ described by Goodman *et al.*²². The oligonucleotides isolated in the fingerprints were extracted from the DEAE paper and the nearest-neighbour distribution of the labelled GMP in the fragments was determined by alkaline hydrolysis. The results for both tRNA $_1^{Tyr}$ and tRNA $_2^{Tyr}$ synthesised *in vitro* are presented in Table 1 and show that given its position on the fingerprint, each oligonucleotide yielded the nearest-neighbour nucleotide expected, assuming it to be derived from tRNA Tyr (ref. 22).

The tRNA $_2^{Tyr}$ transcribed on λ h80T DNA differs from the tRNA $_1^{Tyr}$ species transcribed on ϕ 80 $psu_3^{+/-}$ DNA by the presence of fragment UCACAG (Fig. 3*b*, spot 21). The tRNA $_2^{Tyr}$ fingerprint also contains the fragments ACUG (spot 20) and UAAAψCUG (spot 18), both derived from the anticodon sequence ACUGUAAAψCUG. Because of replacement of G by a C in this sequence in tRNA $_1^{Tyr}$ su_3^+ the fragment is not cleaved by T_1 RNase and appears as oligonucleotide 19 in Fig. 3*a*. As ϕ 80 $psu_3^{+/-}$ DNA codes both for su_3^- and su_3^+ species of tRNA $_1^{Tyr}$, however, the fingerprint in Fig. 3*a* also contains the spots ACUG (20) and UAAAψCUG

(18) derived from su_3^- . The T_1 RNase-resistant fragment C 2'-O-methyl GG, normally found in *in vivo* tRNA Tyr , is absent from both tRNA $_1^{Tyr}$ and tRNA $_2^{Tyr}$ synthesised *in vitro* (Fig. 3*a* and *b*); the CG (spot 3) observed in both fingerprints derives from the unmodified sequence CGG.

To determine the 3'-terminal sequences of tRNA $_1^{Tyr}$ we have synthesised ϕ 80 $psu_3^{+/-}$ RNA using α -³²P-CTP. T_1 RNase fingerprints of α -³²P-CMP-labelled band *e* RNA reveal a spot with a mobility identical to that of the oligonucleotide AAUCCUCCCCACCAOHO observed for *in vivo* tRNA Tyr (ref. 22). The nearest-neighbour distribution of the labelled CMP in this fragment was as expected 2A:2U:7C. Band *d* RNA transcribed on the ϕ 80 $psu_3^{+/-}$ DNA template which migrates in the 5S region of the acrylamide gel (Fig. 1)

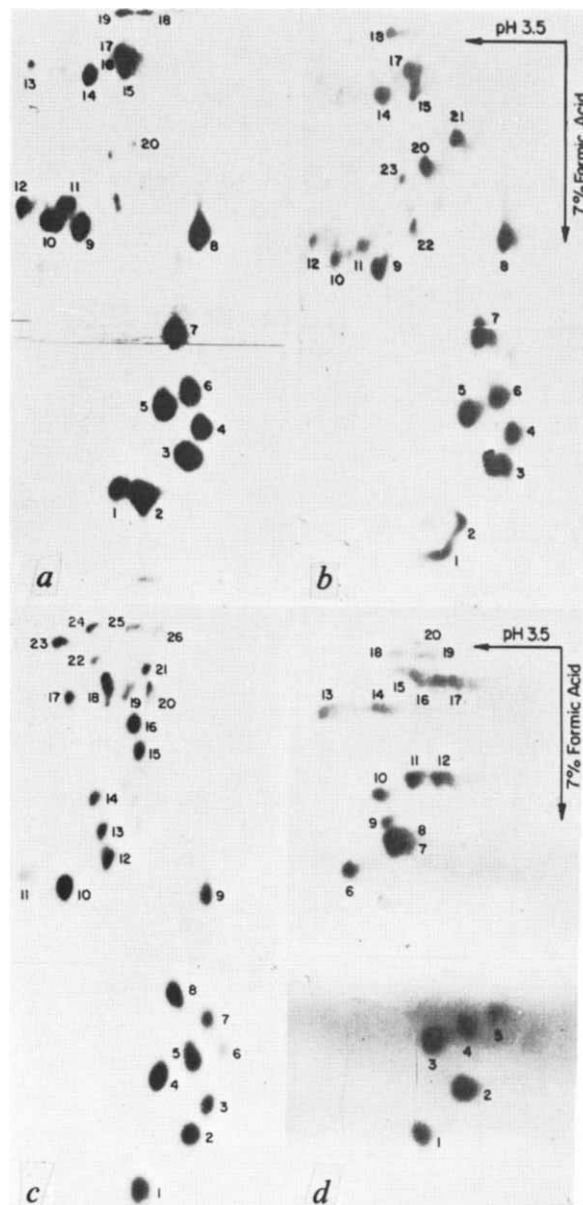


Fig. 3 Autoradiogram of fingerprints of tRNA molecules synthesised *in vitro*. *a*, tRNA $_1^{Tyr}$ (su_3^+ and su_3^-) transcribed on ϕ 80 $psu_3^{+/-}$ DNA (band *e* from Fig. 1). *b*, tRNA $_2^{Tyr}$ transcribed on λ h80T DNA (band *e* from Fig. 2). *c*, tRNA $_1^{Tyr}$ (5S) precursor transcribed on ϕ 80 $psu_3^{+/-}$ DNA (band *d* from Fig. 1). *d*, Mixed fingerprint of tRNA Tyr and tRNA Gly transcribed on λ h80T DNA (band *f* from Fig. 2). Fingerprint analysis was carried out by extraction of the RNA bands from the gel by homogenisation in 0.5 M NaCl, 0.1 M Tris-HCl, pH 8.0, and 0.01 M EDTA, digestion by T_1 RNase and two-dimensional electrophoresis according to Sanger *et al.*²¹.

was also analysed by the fingerprint method. The autoradiogram presented in Fig. 3c identifies this RNA as a $\text{tRNA}_1^{\text{Tyr}} \text{su}_3^+$ precursor. The nearest-neighbour analysis of the T_1 RNase oligonucleotides obtained from the fingerprint separation is similar to that of the *in vivo* 5S precursor of $\text{tRNA}_1^{\text{Tyr}} \text{su}_3^+$ described by Altman and Smith⁷. The fingerprint of the 5S $\text{tRNA}_1^{\text{Tyr}}$ precursor synthesised *in vitro* does not contain the ACUG fragment and seems therefore to be a precursor of $\text{tRNA}_1^{\text{Tyr}} \text{su}_3^+$ only. It contains all the fragments observed in Altman's *in vivo* $\text{tRNA}_1^{\text{Tyr}} \text{su}_3^+$ precursor except the nucleotide pppGp representing the 5' terminus of the molecule. The fingerprint of the *in vitro* product contains in addition small amounts of pGp (spot 11) and a number of unidentified fragments (spots 6, 11–15, 22, 23, 25, 26) which were not observed in the *in vivo* $\text{tRNA}_1^{\text{Tyr}}$ precursor (Fig. 3c).

Transcription of λh80T DNA followed by S100 cleavage of the RNA (Fig. 2) was shown to produce in addition to band *e*, identified as $\text{tRNA}_2^{\text{Tyr}}$, band *f*, which is expected to contain the two tRNA species, tRNA^{Gly} and tRNA^{Thr} . Figure 2 shows that band *g*, present in the transcript even before the processing, behaves like a stable RNA species and migrates on the gel very close to band *f*. It was necessary therefore to repurify band *f* before the fingerprint analysis. The cut-out gel of band *f* was transplanted on top of a 16% acrylamide gel containing 7 M urea and was separated by electrophoresis from the faster running traces of band *g*. The fingerprint of band *f*, (Fig. 3d), seems to contain the mixed sequences of both tRNA^{Gly} and tRNA^{Thr} .

Table 2 Nearest-neighbour analysis of T_1 RNase oligonucleotides from *in vitro* tRNA^{Gly} and tRNA^{Thr}

Spot no.	Corresponding tRNA^{Gly} and tRNA^{Tyr} sequence	Nearest-neighbour predicted	Nearest-neighbour found
1	G	G	G
2	CG(G)	C,G	C,G
3	AG(G)*	A,G	A,G
4	CAG*	A	A
5	CCCG†	C	C
6	UG*	U	U
7	UCG(G)*	C,G	C,G
8	CUG	U	U
9	DAG*	A	A
10	AUG†	U	U
11	UAAG(G)*	A,G	A,G
12	CAUCG†, CUCAG*	C,A	C,A
13	DDG(G)*	U,G	U,G
14	TvCG	C	C
15	AUAUAG*	A	A
16	AUUCG*, AAUCUG*	C,U	C,U
17	CCUAUCAG*, CACCCUUG(G)†	A,G,U	A,G,U
18	UAUAAUG(G)†	U,G	U,G
19	CUAUUACCUCAG†	A	A
20	CCUUCUAAG†	A	A

* Fragment derived from tRNA^{Thr} (ref. 24).

† Fragment derived from tRNA^{Gly} (ref. 23).

In Table 2, the nearest-neighbour analysis of the different fragments from the fingerprint is compared with that expected for the respective *E. coli* tRNA species^{23,24}. The T_1 oligonucleotide 7^mUGC²⁴ is absent from the fingerprint of the tRNA^{Thr} synthesised *in vitro*. Due to the presence of a non-modified G at the 5'-terminus, this fragment is degraded by T_1 to form UCG which appears as spot 7. Note that the pGp fragment, normally derived from the 5'-terminus of the tRNA^{Thr} and tRNA^{Gly} (refs 23 and 24) is absent from the fingerprint of the *in vitro* tRNAs synthesised *in vitro*. A tentative explanation for the absence of pGp at the 5'-terminus of *in vitro* tRNA^{Thr} and tRNA^{Gly} may be a wrong precursor cleavage by RNase P.

The RNA molecules migrating as band *g* (Fig. 2) were also

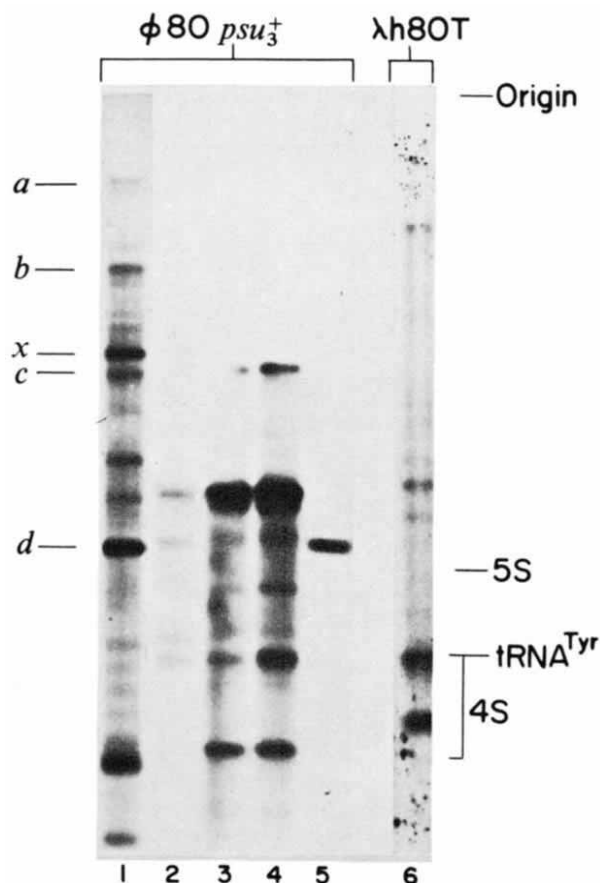


Fig. 4 Autoradiogram showing formation of tRNA from isolated precursor RNA. RNA bands were extracted from the gel separation of $\phi 80 \text{psu}_3^{+/-}$ RNA or λh80T RNA synthesised *in vitro*, incubated for 60 min with S100 extract (10 min for band *a* from Fig. 1) and fractionated by polyacrylamide gel electrophoresis: 1, 2, 3, 4 and 5 refer to bands *a*, *b*, *x*, *c* and *d* from Fig. 1; 6 refers to band *a* from Fig. 2.

studied by the fingerprint method but did not seem to contain tRNA sequences.

Polycistronic tRNA precursors

Figure 1 suggests that RNA band *a* is a primary transcription product of the $\text{tRNA}_1^{\text{Tyr}}$ genes on $\phi 80 \text{psu}_3^{+/-}$ DNA and that bands *b*, *x*, *c* and *d* are intermediate precursors of tRNA^{Tyr} . To provide further support for this assumption we have extracted the RNA bands from the gel and subjected them to cleavage by incubation with S100 extract. Figure 4 shows that a short incubation (10 min) of band *a* with S100 extract generates the intermediate RNA bands *b*, *x*, *c* and *d* as well as some tRNA^{Tyr} . RNA bands *b*, *x* and *c* all produce tRNA^{Tyr} band on processing. Although band *d* RNA has been shown by fingerprint analysis (Fig. 3c) to carry tRNA^{Tyr} sequences it was not cleaved after extraction from the gel and incubation with the S100 extract (Fig. 4). This behaviour is unexpected in view of the fact that this band does not seem to accumulate significantly when processing by S100 extract is carried out directly in the transcription mixture (legend to Fig. 1). The possible reasons for this observation are being investigated.

The isolation and cleavage of band *a* RNA from λh80T DNA transcript (Fig. 2) generates two RNA bands in the 4S region of the gel, one corresponding to tRNA^{Tyr} and the other to the mixture tRNA^{Gly} , tRNA^{Thr} (Fig. 4, gel 6). It may therefore be concluded that the three tRNA species coded by λh80T DNA are present on a common polycistronic precursor. Polycistronic precursors for the tRNA gene clusters carried

by $\phi 80psu_3^{+-}$ DNA and $\lambda h80T$ DNA have not yet been shown *in vivo*. The dicistronic RNA molecule containing tRNA^{Gly}, tRNA^{Thr} isolated by Carbon⁶ may be only a part of a primary precursor. The transcription *in vitro* of the adjacent genes of tRNA^{Tyr}, tRNA^{Gly}, tRNA^{Thr} and tRNA^{Tyr} (su_3^+ and su_3^-) as polycistronic precursors provides direct evidence that these genes belong to a single transcriptional unit.

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Is the evolution of insulin Darwinian or due to selectively neutral mutation?

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A model for the evolution of insulin mainly in terms of adaptive processes is discussed. The model depends critically on the relationship of sequence changes to the three-dimensional structure and the role of various parts of this structure in the conversion of the proinsulin molecule to the active form, the storage of insulin, its transport to the site of action and its interaction with a receptor.

SEQUENCES of functionally equivalent proteins provide the best evidence for selectively neutral mutations. As the knowledge of proteins increases, however, more detailed attempts to discern the relative importance of adaptive and neutral mutations in evolution may become possible. In particular, the detailed study of the role of different amino acid residues in a class of proteins and the effects of sequence changes on their physical and physiological properties is an essential prerequisite to an assessment of arguments for and against neutral mutations^{1,2}. Three principal arguments are given in support of neutral mutations. Kimura³ suggested that the substitutional load due to the high rate of evolution would have been intolerable unless most of the mutations had been neutral in natural selection. The main argument of King and Jukes⁴ is based on the observation that the rates of evolution of individual proteins are uniform and the idea that most amino acid differences between species are functionally insignificant. This uniform rate and the apparent latitude for random genetic change at the molecular level is said to hold for cytochrome *c*, haemoglobin, histone and also for insulin with the exception of guinea pig and coypu insulins. Markussen⁵ has calculated evolutionary rates for insulins based on nucleotide differences and considered insulin evolution in terms of predominantly neutral mutation.

We feel that the above views result from an oversimplification of the meaning of 'functional insignificance' and depend on the use of average rates of change without sufficient regard to the location and chemical nature of

sequence changes. We wish to discuss a model for the evolution of insulin mainly in terms of adaptive processes. We do not exclude the possibility of some neutral change; rather we wish to provide an alternative explanation for the evolution of insulin and to caution conclusions made on the basis of uniform rates and changes that seem not to not affect function.

Insulin is a protein hormone produced and stored in the B cells of the islets of Langerhans of the pancreas. It is released into the circulation from membrane-limited intracellular storage granules in response to raised blood glucose levels and facilitates removal of glucose and other metabolites to the tissues. Insulin is synthesised as a single chain precursor—proinsulin. Within the storage granules, enzyme action removes a peptide about 30 residues long—the connecting peptide—leaving the active hormone. Most insulins comprise 51 amino acids, 21 in the A chain and 30 in the B chain. The chains are linked by two disulphide bonds. Insulin binds to specific receptor sites on the target cell surface but the details of how a biological response is initiated are not fully understood.

The amino acid sequences of 28 insulins (from the jawless hagfish through bony fishes such as cod and birds such as the turkey, to mammals) have been determined (Table 1) and these insulins are active in other test animals. The rate of amino acid substitution for most insulins has been in the order of 1×10^{-8} per locus per year (Paulings).

The three-dimensional structure of pork insulin has been described in detail by Hodgkin *et al.*^{7–9}. The biosynthesis of proinsulin¹⁰, the storage in the B cell granules^{11,12} and the receptor binding^{13–15} have been studied widely. Our views depend critically on the relation of sequence changes to the three-dimensional structure and the role of various parts of this structure in the different aspects of the physiology of insulin. In fact several characteristics of insulin may be of importance in determining fitness. These might include the correct folding of the newly synthesised proinsulin molecule and its conversion to an active form, the storage of insulin, its transport to the site of action and its interaction with a receptor. Most of these characteristics

Table 1 Insulin sequences

A chain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31			
	Gly	Ile	Val	Glu	Gln	Cys	Cys	Thr	Ser	Ile	Cys	Ser	Leu	Tyr	Gln	Leu	Glu	Asn	Tyr	Cys	Asn	—										Pig		
								Ala		Val																						Cattle		
								Ala	Gly	Val																						Sheep		
								Gly																									Horse	
								Ala		Thr																						Sei whale		
				Asp																													Rat	
									Gly	Val																							Elephant	
				Asp					Gly	Thr		Thr	Arg	His			Gln	Ser														Guinea pig		
				Asp					Asn				Arg	Asn			Met	Ser				Asp										Coypu		
												Thr																					Chinchilla	
																																	Cod	
								His	Arg	Pro		Asp	Ile	Phe	Asp		Gln															Angler		
								His	Arg	Pro		Asn	Ile	Phe	Asp		Gln															Tuna 2		
								His	Lys	Pro		Asp	Lys	Phe	Asp		Gln	Ser															Toadfish 2	
								His	Arg	Pro		Asp	Ile	Phe	Asp		Gln	Ser															Toadfish 1	
			His					His	Lys	Pro		Asp	Ile	Phe																			Bonito	
								His	Lys	Arg			Ile		Asn		Gln																Hagfish	
								His	Asn	Thr																							Turkey	
								Glu	Asn	Pro																							Duck	
B chain	Phe	Val	Asn	Gln	His	Leu	Cys	Gly	Ser	His	Leu	Val	Glu	Ala	Leu	Tyr	Leu	Val	Cys	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Pro	Lys	Ala		Pig		
																																	Elephant	
																																	Rabbit	
			Lys						Pro																								Rat 1	
			Lys																														Rat 2	
			Ser	Arg						Asn				Thr			Ser			Gln	Asp	Asp					Ile		Met	Ser		Guinea pig		
	Tyr		Ser		Arg					Gln			Asp	Thr			Ser			Arg	His			Tyr	Arg	Pro	Asn	Asp	—			Coypu		
																																	Chinchilla	
			Lys										Asp								Asp								Met					
	Met	Ala	Pro	Pro									Asp								Asp						Asn		—				Cod	
	Val	Ala	Pro	Ala									Asp								Asp						Asn		—				Angler	
	Met	Ala	Pro	Pro									Asp								Asp						Asn	Ser	—	—			Toadfish 2	
		Ala	Ala		Pro																						Gln						Bonito	
	Val	Ala	Pro	Pro									Asp								Asp						Asn		—				Tuna 2	
		Arg	Thr	Thr	Gly					Lys	Asp		Asn				Ile	Ala			Val						Asp		Thr	Lys	Met		Hagfish	
	Ala	Ala																										Ser					Turkey	

Mammals

Fish

Birds

Mammals

Fish

Birds

will depend on the integrity of the three-dimensional structure, possibly on the quaternary structure and certainly on the solubility of insulin, its interaction with other proteins in the organism, its thermodynamic stability and its resistance to enzymatic degradation. All these aspects could act as restraints at various stages of evolution and changes in the anatomy and physiology of different animals may change these restraints. For instance, the observation¹⁰ that the location of the insulin-producing B cell has moved from the gut wall in tunicates to clusters of cells at the anterior end of the gut in Agnatha and further to the specialised organ, the pancreas, enclosing both endocrine and exocrine (especially proteolytic) functions in higher vertebrates may give us some clue to the changes that could have occurred in storage restraints on the insulin molecule.

For insulin we can now begin to define the above characteristics in terms of the tertiary structure and the amino acid sequences. In this discussion we include the unpublished results of X-ray and circular dichroism studies together with the biological properties of a number of different insulins of known sequence.

Structure and function

X-ray analysis shows that the pig insulin monomer has a well defined, globular three-dimensional structure with an hydrophobic core and two predominantly hydrophobic surfaces (Fig. 1). These surfaces are important for association of the insulin molecules first as a dimer and then in the presence of zinc to a hexamer. The surface of the hexamer contains most of the hydrophilic residues. The hexamer has the shape of a torus with two zinc atoms bound in the central, cylindrical channel (Fig. 2). The amino acids responsible for receptor binding and stimulation of a biological

response are located by studies of chemically modified and semi-synthetic insulins. The monomer is the active form; although receptor binding involves some of the hexamer surface it probably requires certain of the hydrophobic residues involved in dimerisation also (Fig. 1). Maintenance of the tertiary structure seems to be essential for receptor binding.

The connecting peptide of proinsulin links residues A1 and B30 (Fig. 1) which are only 10 Å apart although the peptide is about thirty amino acids long. The proinsulin molecules hexamerise¹⁷, thus the connecting peptide must lie on the hexamer surface. The role of the connecting peptide seems to be not only concerned with the folding of proinsulin to the correct tertiary structure but also to increase the solubility of the hexamer in the presence of high zinc concentrations¹⁸. When the connecting peptide is cleaved the insulin crystallises as granules; with the exception of some hystricomorph insulins and probably hagfish insulin, the granules contain zinc insulin hexamers. The role of the zinc insulin hexamers seems to be to provide a storage form which is thermodynamically stable and more resistant to enzymatic degradation than the unassociated form. The zinc-containing granules of different species are usually crystalline in appearance but electron microscopy shows that they have a varied morphology, symmetry and molecular packing^{11,12}. This variation in granule structure finds a parallel in the variation of crystal shapes and forms in the laboratory. It seems that the ease of crystallisation of zinc insulin hexamers may be a reflection of the granular structure found in the B cell, and the insulin that can form zinc insulin hexamers may have evolved as a stable storage form. The hexamers are probably unrelated to receptor binding. Thus it can be seen that there are many facets of

Variable residues on surface
of hexamer

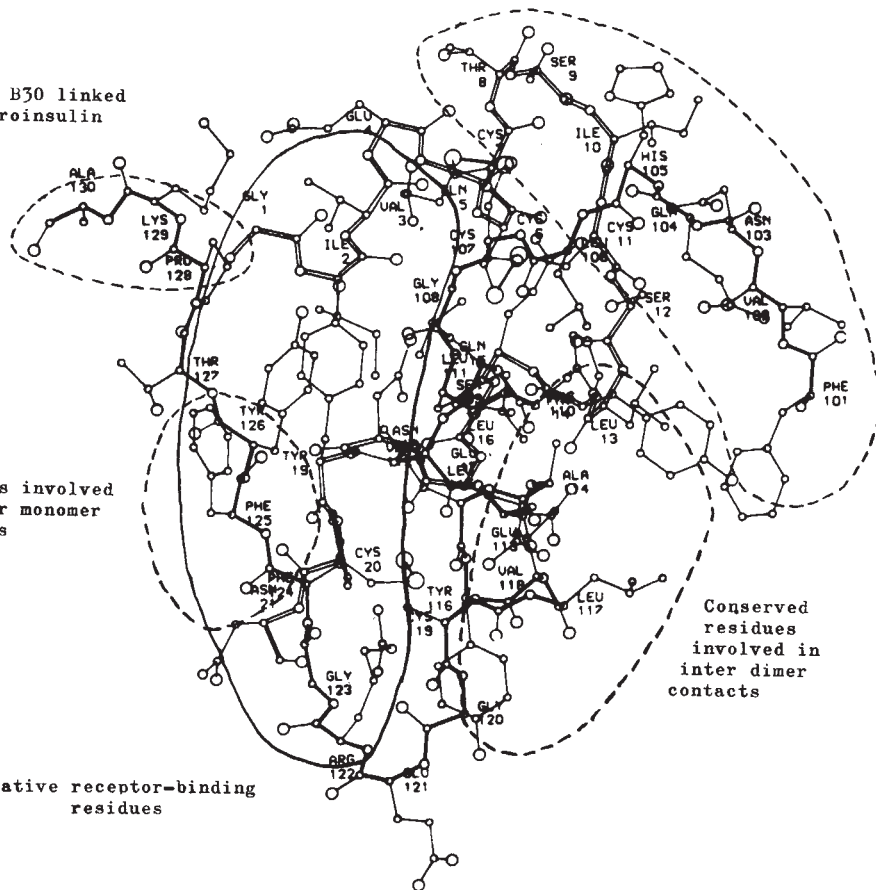
A1 and B30 linked
in proinsulin

Residues involved
in inter monomer
contacts

Putative receptor-binding
residues

Conserved
residues
involved in
inter dimer
contacts

Fig. 1 View of the insulin monomer indicating how the surface may be described in terms of various areas involved in activation from the prohormone, binding to the receptor and self association.



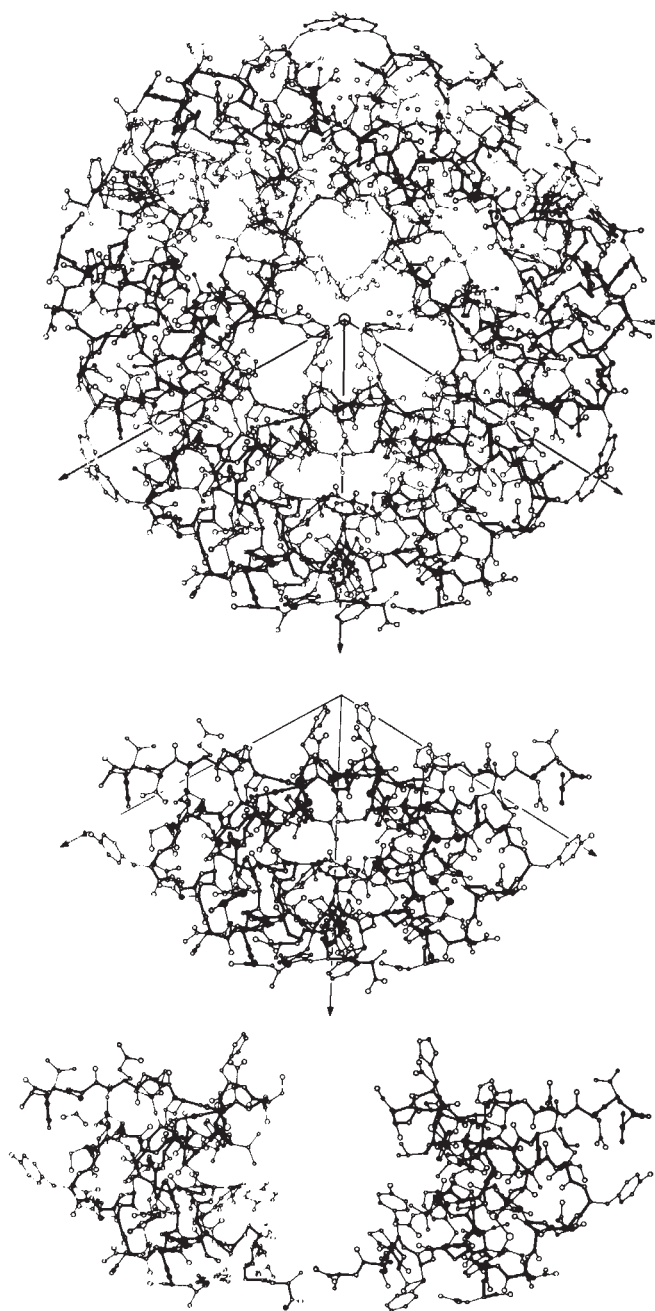


Fig. 2 Formation of the zinc insulin hexamer.

adaptation in the insulin molecule, only one of which is receptor binding.

Sequences and structure

The sequences available indicate that all insulins might attain the tertiary structure found for pig insulin. The disulphide bridges and the hydrophobic core of the monomer are invariant. The residues which vary do not have the same location in different classes of animals. In the mammals, with the exception of the hystricomorphs, all insulins can form dimers and zinc insulin hexamers. The most highly variable residues are A8–A10 and B29 and B30. These residues are close together on the hexamer surface; they are not essential for formation of the zinc insulin hexamer, but they do affect its solubility and its

ability to crystallise. Pig insulin molecules pack in the crystals with contacts between two equivalent isoleucine A10 residues. In bovine insulin the A10 residues are valines. As a consequence the crystal packing of the hexamers is slightly different as is shown by a 2.3 Å difference electron density map. The faces of bovine insulin crystals grow at different rates from those of pig insulin crystals and thus the crystals have a rather different appearance (Fig. 3). Such changes of solubility and crystal form may affect granule formation; although the changes are only small they may reflect the differences in granules of different mammals and these may have functional significance. We cannot rule out the possibility that the nature of B30 is important to a species-specific proinsulin conversion enzyme, but it does seem that the changes at A8–A10 and at B30 found in mammals have little effect on the receptor binding.

Rats show polymorphism in their insulins, and rat insulin I is unique in that it contains a proline at B9, an otherwise invariant residue. The two rat insulins have similar biological activities. The rat I insulin, however, has a rather different dependence of its circular dichroism on zinc concentration (T.L.B., S.P.W., and A. Wollmer, unpublished). This indicates that the proline at B9 may decrease the association constant to zinc hexamers. Thus, this change at B9 may not be selectively neutral in terms of aggregation of insulin in the storage process, although it seems that given sufficient zinc, rat I like rat II insulin can crystallise as zinc hexamers. The packing relationships of the hexamers in crystals show very different symmetries for the two insulins. Rat I insulin crystals are rhombohedral whereas rat II insulin hexamers pack in a cubic lattice, space group $P4_232$ (Fig. 3). This cubic crystal form may be related to the unusually clear crystalline arrangement of some rat granules shown by electron microscopy.

For fishes the nature and location of the changes is different and certainly not random. The residues A8, A9 and A10 are very different from those found in mammals and the variation within fishes is much less. In particular A8, A9 and A10 are conserved in fishes as histidine-basic proline residues; in mammals they are smaller, non-basic and sometimes hydrophobic. In contrast to the mammals, residues A12–A15, A17 and B1–B3 are changed in fishes. Most also possess an extra B-chain residue at the amino terminus. These two groups of residues (especially B0, B1, A13, A14, A17) are not randomly distributed but are close together in the monomer and are clustered with equivalent residues when dimers aggregate to hexamers. The changes are complementary in that they still allow zinc hexamer formation. A single change of the kind observed in most of these locations would not favour hexamerisation. Although collectively these changes do not affect hexamer formation, they do affect the nature of the crystal packing and therefore perhaps granulation. In the laboratory cod insulin can be crystallised in conditions used to prepare pig insulin crystals but the crystal form is orthorhombic instead of rhombohedral, although the crystals also contain zinc insulin hexamers¹⁹. The sequence changes seen in the insulin of the jawless hagfish, however, do affect aggregation. In this case the zinc binding residue B10 is not histidine and the insulin does not form zinc insulin hexamers. The storage form must be radically different, although there may be some aggregation through non-polar surfaces²⁰. The hagfish insulin may resemble an ancestral insulin²¹ which was not subject to severe storage restraints and thus had not evolved the complex enzyme-resistant, storage mechanism typical of mammals.

The insulins of rodents from the suborder Hystricomorpha are different from the insulins discussed above as they do not show the uniform rate of evolution characteristic of the other insulins. The insulins of the guinea pig and coypu have changed at B10. The residue is no longer the zinc-binding histidine, but rather asparagine or gluta-

mine. Ultracentrifuge²² and circular dichroism²³ studies indicate that guinea pig insulin does not hexamerise in the presence of zinc. Examination of the changes in amino acid sequences of coypu and guinea pig insulins show that there are further changes related to this lack of association. Residues B14, B17 and B20 uniquely change in guinea pig and coypu insulins to larger and more hydrophilic residues. Residues B4 and A1 also change to radically different residues; they are larger and more basic. All of these residues are clustered together in the three-dimensional structure and make up the large surface which in other mammals is hydrophobic and involved in close contacts in the zinc insulin hexamer. The residue changes in the guinea pig and coypu are not only non-randomly located, they also make the surface more hydrophilic and thus the insulin molecule more thermodynamically stable in the absence of aggregation in aqueous environments. The relevance of this to storage is emphasised by the finding that guinea pig granules contain no zinc and do not have a crystalline appearance. Both guinea pig and coypu insulins also have changes in the B-chain carboxy terminus. In guinea pig insulin the change is at B22 (arginine to glutamic acid). B22 arginine is normally involved in an iron pair interaction with the carboxy terminus of the A chain. In bovine insulin, removal of the carboxy terminus by the action of carboxypeptidase destroys this stabilising ion pair; the tertiary structure is very disturbed and the insulin no longer dimerises. In a similar way, circular dichroism shows that guinea pig insulin has a changed tertiary structure and cannot dimerise²¹ (Fig. 4). Furthermore, guinea pig insulin has a very low biological activity, about 2% of that of bovine insulin in *in vitro* assays.

Evolution of insulins

These observations allow us to construct some hypotheses concerning the evolution of insulins. We will first consider guinea pig insulin. Let us suppose that there was a local shortage of zinc to the B cell in guinea pig ancestors. Insulin would not be aggregated with the same ease to zinc insulin hexamers. It would then be selectively advantageous to allow mutations which make the surface involved in aggregation to hexamers more hydrophilic to increase

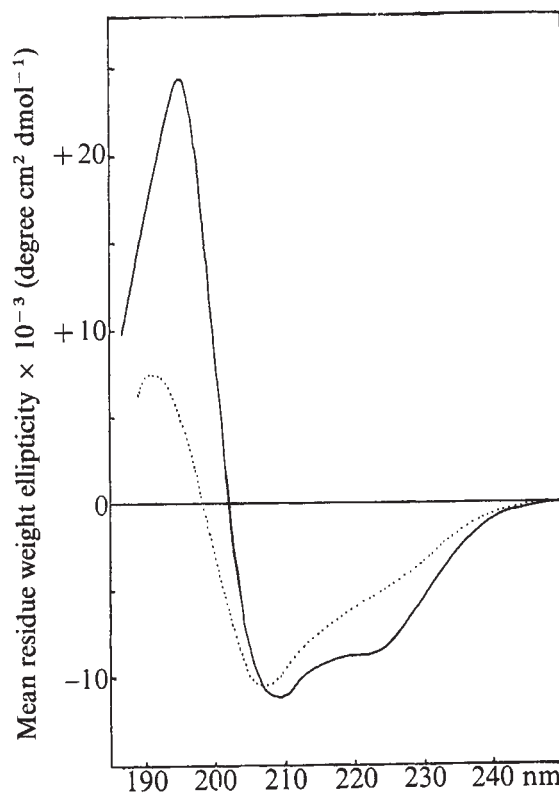


Fig. 4 Far ultraviolet circular dichroism spectra of bovine (—) and guinea pig (·····) insulin showing considerable differences in solution conformation.

thermodynamic stability. Although thermodynamic stability is increased, however, the unaggregated insulin molecules are still more susceptible to enzymatic degradation than zinc hexamers. It is well established that zinc insulin hexamers are much more resistant to tryptic digestion than the less associated forms. Thus it may be that the change of the trypsin-sensitive arginine at B22 to glutamic acid is selectively advantageous in terms of stabilising the molecule

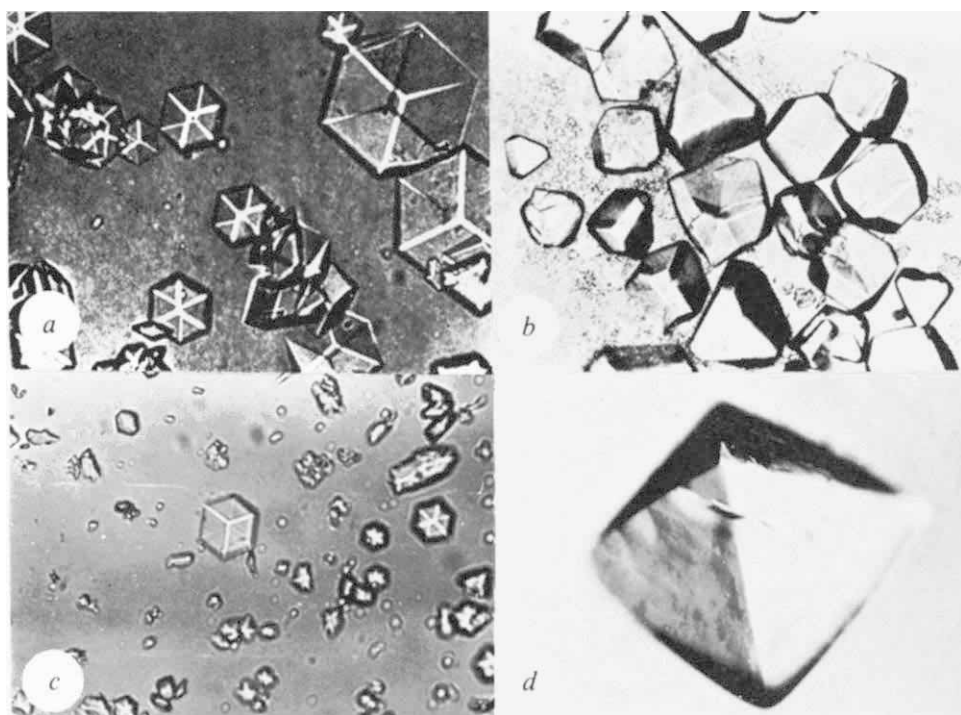


Fig. 3 Two Zn insulin crystals. a, Bovine; b, porcine; c, rat I; d, rat II.

to enzymatic degradation²². The changes, however, cause a change of tertiary structure, and this no doubt largely contributes to the decrease in binding affinity of the guinea pig insulin to adipocyte receptors. In fact it has been established that guinea pig insulin is less active even in guinea pigs compared with bovine insulin⁶. This implies that guinea pig insulin has evolved to maximise its stability in storage but this has been deleterious as far as receptor binding is concerned. We have to assume that the role of storage is as important to the fitness of the organism to survive as is the primary function, that of receptor binding and biological activity. In summary the arguments concerning the guinea pig insulin seem to implicate adaptive changes related not to receptor binding but rather to several other characteristics which are important to the physiological role of the insulin molecule. This is consistent with the high rate of acceptance of mutations in this and possibly some related species²⁴.

For other insulins the rate is less but uniform and this has been said to be evidence in favour of neutral mutations. Neutral mutations would be expected to occur randomly within certain areas of the molecule which are not affected by restraints. Our description of the location of the amino acid changes, however, has demonstrated that the location of accepted mutations has been distinctly different in different classes of animals. We have shown that these changes affect different physical properties such as three-dimensional structure, solubility, thermodynamic stability, resistance to enzymatic degradation, self-association and crystallisation to different extents. Furthermore, these changes will probably affect various aspects of the physiology such as activation of the proinsulin molecule, the nature of the storage of insulin in granules and its transport to the storage site and to the receptor apart from any effect on the receptor binding. It is therefore possible that they may also have been adaptive changes. Although the general nature of the insulin receptor binding has been retained during evolution, the nature of these other physiological restraints may have changed at different times. But how does this give rise to the apparent constant rate of amino acid substitution?

If there are a number of different restraints on the acceptance of mutations, a change of restraint will necessarily give rise to a noticeable change in the rate of acceptance in the following circumstances. First, if a change of restraint is very rare and occurs at only one or two points in evolution. Second, if changes in the insulin molecule are advantageous in terms of one characteristic but lead to a decrease in fitness in terms of other characteristics. Third, if by chance the restraints all changed in one phase of evolution due to changes in selective pressure on the organism as a whole. In the case of insulin, let us assume that adaptive changes dominate observed changes in evolution, but that the nature of the restraints has been different over different periods of time during evolution. This would be consistent with the several different locations of accepted mutations in insulins from different classes of animal. In the case of guinea pig insulin, the restraints are not independent in our model. Certain of the amino acid changes are involved in more than one aspect of function. Inability to bind zinc affects thermodynamic stability, susceptibility to enzymatic degradation and receptor binding of the insulin. Thus the restraints are interdependent and further mutations beneficially affecting each of these restraints become selectively advantageous. This gives rise to a cascade of evolutionary change.

In the other insulins we have no reason to suspect that changes which are adaptive in terms of one characteristic have serious consequences for other aspects of the physiology of insulin. Thus a change in the residues affecting crystallisation may be advantageous in terms of stability of the granules or the rate that they are dissolved in the circula-

tion, but it may not seriously affect the rate of activation of the proinsulin molecule, the receptor binding or thermodynamic stability. Thus the changes in restraint do not usually lead to a cascade effect, and they may be considered as independent variables. If the changes of restraint are frequent in evolution but independent, there is no reason why we should observe a sudden change in the rate of evolution. Statistically it is not unlikely that the changes of restraints (if there are a large enough number of them) will give rise to a fairly uniform rate of change in amino acid sequence, although more detailed examination of the changes would reveal their location in the protein not to be random. This is what is observed for most insulins.

This argument assumes that the total pressure on the molecule at any one time is roughly constant. This at first seems to be an unlikely assumption. But, if the restraint changes which give rise to adaptive mutations do not directly relate to major aspects of the physiology of the animals, then this assumption seems more reasonable. For instance, changes in the solubility of the granules and the stability of the insulin molecules in circulation may be related to rather sophisticated changes in the speed of response to insulin and to the time during which an insulin molecule is active before it is degraded and removed from the system. Changes of this nature in quite closely related species are not unreasonable and may increase the fitness of the animal to its environment. Furthermore, some adaptive changes may occur to eliminate undesirable situations such as chance interaction of the insulin molecule with other proteins within the cell and during circulation.

In the insulin molecule we have seen that the receptor binding is a fairly constant restraint during evolution, and this has led to an invariance of some surface residues and those residues in the hydrophobic core which are responsible for the general tertiary structure and consequently the proper arrangement of the residues binding the receptor. The number, nature and relative importance of restraints will differ from protein to protein and so the average rate of acceptance of mutation would be expected to vary although in many cases it might be relatively constant within a group of functionally equivalent proteins. For instance, in the proinsulin-connecting peptide most residues must be on the surface of the proinsulin molecule and therefore they will be more susceptible to variations in the environment of the protein. The connecting peptide must also accommodate changes in the insulin molecule whose surface it covers. It is therefore plausible that the characteristics which act as restraints on the acceptance of mutations change more frequently for the connecting peptide. This is consistent with the higher rate of change of this peptide than for the insulin molecule as a whole.

In conclusion, it seems that in rare cases an amino acid may be changed in a way that is advantageous to one characteristic but deleterious to others. The functional characteristics are interdependent and the rate of evolution will undergo a sudden burst. A uniform rate for most proteins is, however, not unexpected if the functional restraints are independent in terms of the amino acids which are affected. A uniform rate of evolution and the observation that amino acid changes are functionally insignificant with respect to one characteristic do not seem to be conclusive arguments for attributing most sequence variation observed in proteins to selectively neutral or non-Darwinian evolution.

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letters to nature

Precession in transient X-ray sources

THE origin of both the relatively long period and the transient nature of the recently discovered X-ray sources Ariel 1118–61 (ref. 1) and Ariel 0535+26 (ref. 2), with pulse periods of about 405 and 104 s, respectively, and decay times of days, is unclear. The pulse rate of A1118–61 has been attributed to an extremely short binary star orbital period³, or to an extremely long neutron star rotation period⁴. The quasi-sinusoidal nature of both X-ray light curves, unlike the sharply pulsed flux from Her X-1 and Cen X-3, would seem difficult to account for in a rotation model. The 104-s period, on the other hand, seems embarrassingly short for a binary orbital period. Therefore, I reiterate here an earlier suggestion⁵ that free precession may be important in such galactic X-ray sources. Such precession, which provides a plausible explanation for the 35-d quasi-sinusoidal period and intensity variation of Her X-1 (which has a 1.25-s pulse period) could directly account for the shape of the light curves of the transient X-ray sources.

If, as has been suggested, the initial X-ray outburst is triggered by the close approach of a neutron star (or white dwarf) to its main sequence companion, either because the collapsed star is moving in a highly elliptical orbit⁶, or because the radius of the companion is variable⁷, the accompanying rapid mass transfer might well provide the torque necessary to excite free precession of large amplitude in the collapsed object. For a neutron star of mass M , surface radius R , Alfvén surface radius r and total mass accreted m , the spin axis could change direction by an angle $\theta = mr^2/MR^2$ during a single encounter. The superposition of the effect of many mass transfer events (even if it is stochastic) could easily lead to a precession angle of large amplitude ($\theta \approx 10$ – 100°), since the damping times can be long (for example, greater than 10^6 yr for neutron stars⁸).

For a star spinning with a period T_s , and having matter density ρ , the free precession period T_f is roughly $T_f \approx \alpha T_s^3 G \rho$, where α is a numerical coefficient of order unity which depends on the detailed structure of the collapsed object (density distribution, rigidity, and so on). Identifying the observed periods of a few hundred seconds with the free precession period of a neutron star ($\rho \approx 2 \times 10^{14}$ g cm⁻³) and taking $1 \gtrsim \alpha \gtrsim 10^{-2}$, one finds $T_s \approx 0.1$ – 0.01 s. This range of spin periods is plausible, encompassing as it does the periods of three known pulsars (the Crab, binary and Vela pulsars with pulse periods of 0.033, 0.059, and 0.089 s, respectively). Such a high spin rate need not suppress matter accretion from a disk, provided that the magnetic fields of the neutron stars are somewhat smaller than the usually assumed value of 10^{12} gauss. The discovery of regular short-period pulsations

or amplitude variations in the X-ray flux from these transient sources would offer strong support for the present suggestion; its absence, although difficult to reconcile with most plausible beam profiles, is not decisive.

White dwarf X-ray sources, with spin periods of order 100 s, on the other hand, could also show evidence of free precession, but with a period of days. One might expect such a period to show up in the mean (averaged) intensity variation over an observation period of weeks. (I note that at least one white dwarf showing circular polarisation and thus probably having a strong surface magnetic field, G195–19, shows regularly periodic variations in polarisation with a period of 1.33 d (ref. 9), a period the discoverers attribute to rotation but which could well be the free precession period arising from a spin period of about 100 s.) In the present context, the discovery of either short (0.1-s) or long (1-d) periods in any of these X-ray sources could distinguish between models involving white dwarfs and those involving neutron stars.

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Possible cosmological origin of spontaneous symmetry breaking

POPULAR unified theories of weak and electromagnetic interactions¹ are based on the notion of a spontaneously broken gauge symmetry. The hope has also been expressed by several authors that suitable generalisations of such theories may account for strong interactions as well. Here we propose that the spontaneous breakdown of gauge symmetries may have a cosmological origin. As a consequence we find that at some early stage of development of an expanding universe, a phase transition takes place. Before the phase transition, weak and

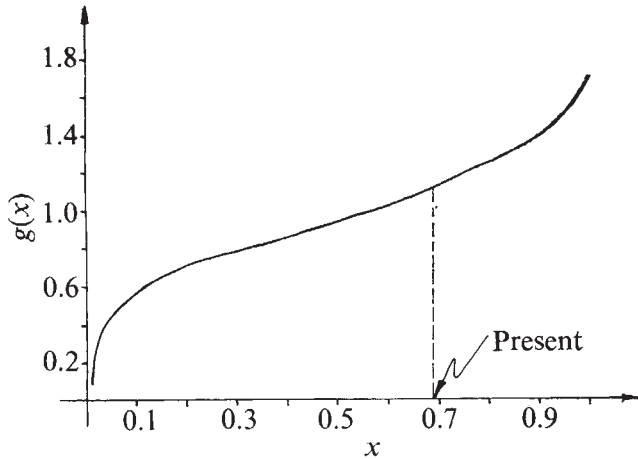


Fig. 1 Vacuum expectation value of the Higgs field plotted against the expansion parameter of a "standard" universe.

electromagnetic interactions (and perhaps strong interactions too) are of comparable strengths. The presently observed differences in the strengths of the various interactions develop only after the phase transition takes place.

In order to illustrate our ideas, we consider a 'proto-type gauge theory' of the Salam-Ward-Weinberg variety (with gauge group $SU(2)_{\text{LEFT}} \otimes U(1)_{\text{RIGHT}}$) in a curved space with prescribed metric tensor $g_{\mu\nu}(x)$. The action functional of the theory may be written as $W = W_0 + W_H$, where W_0 stands for the gauge-invariant action of massless Fermion fields and of the gauge fields. The form of W_0 is well known in flat space-time¹ and it is a trivial exercise to generalise that expression to a space of any given metric tensor $g_{\mu\nu}$. The action W_0 is automatically invariant under (infinitesimal) Weyl transformations, $\delta g_{\mu\nu}(x) = 2\Lambda(x)g_{\mu\nu}(x)$, $\Lambda(x)$ being an arbitrary smooth function. Likewise, the action of the isodoublet Higgs field, W_H , can be written in a form which is invariant under Weyl transformations^{2,3}:

$$W_H = \int d^4x \sqrt{-g} \left\{ -\frac{1}{2} g^{\mu\nu} \Phi^\dagger \left(\partial_\mu + i \frac{g'}{2} B_\mu + i \frac{g}{2} \tau A_\mu \right) \Phi + \left(\partial_\nu - i \frac{g'}{2} B_\nu - i \frac{g}{2} \tau A_\nu \right) \Phi - \frac{1}{12} R(\Phi^\dagger \Phi) - \frac{\lambda}{24} (\Phi^\dagger \Phi)^2 \right\} \quad (1)$$

Unless stated otherwise, we follow the notation of ref. 1 with obvious modifications to accommodate an arbitrary space-time metric. The quantity R stands for the Ricci scalar, $R = g^{\mu\nu} R_{\mu\nu}$. So Weyl (conformal) invariance can be broken only by the properties of the "cosmic background". In this approximation, our model is consistent with Mach's principle.

We assume that the metric is of a Robertson-Walker form,

$$ds^2 = dt^2 - a(t)^2 [(1-r^2)^{-1} dr^2 + r^2 d\Omega^2]$$

corresponding to a "standard" cosmology⁴, t being the "cosmic time", and so $a(t)$ is the radius of the universe. Units are chosen such that $\hbar = c = L = 1$ where L stands for the Planck length. We re-emphasise: the influence of the matter fields on the metric is neglected in this model. This implies that the energy-momentum tensor of matter can be written as $T_{\mu\nu} = p g_{\mu\nu} + (\rho + p) u_\mu u_\nu$, where p , ρ , u_ν are the classical pressure, mass-energy density and four-velocity, respectively.

Spontaneous symmetry breaking develops if the vacuum expectation value (VEV) of the neutral Higgs field, $\phi = \frac{1}{2} \times (1 - \tau_3) \langle \Phi \rangle$ differs from zero. In analogy with the flat-space case, ϕ satisfies the equation $(\square + (R/6))\phi + (\phi^3 \lambda/6) = 0$. To qualify as the VEV of a quantised field, ϕ can at most depend

on the cosmic time, t . Using the classical equations of the standard cosmology, $da = [(8\pi a^2/3) - 1]^{1/2} dt$, one introduces the expansion parameter $x = a(t)/a_{\text{max}}$ as an independent variable. The dimensionless parameter λ can be 'scaled out' by introducing the function $g = (\lambda a_{\text{max}}^2)^{1/2} \phi$. In the spirit of the standard cosmology, we assume that for $x \lesssim x_0$ the "matter" distribution is extremely relativistic ($p \approx (\rho/3) \approx 0$), while for $x \gtrsim x_0$ the distribution is "pressureless", $p \approx 0$, $\rho \approx x^{-3}$. Thus, x_0 stands for the expansion parameter at which the "big bang" universe cools down sufficiently so as to become a "quiet" universe following a "fireball" stage. The value of x_0 is generally estimated to lie between the limits $10^{-6} \lesssim x_0 \lesssim 10^{-3}$, whereas the "present" value of x is approximately $x_1 \approx 0.69$. Corresponding to $p \approx \rho/3 \approx 0$ ($x < x_0$), and $p \approx 0$, $\rho \approx x^{-3}$ ($x > x_0$), we deduce,

$$\left(\frac{1-x^2}{x^2} \right)^{1/2} \frac{d}{dx} \left[x^3 \left(\frac{1-x^2}{x^2} \right)^{1/2} \frac{dg}{dx} \right] + \frac{x^3}{6} g^3 = 0 \quad (x < x_0)$$

$$\left(\frac{1-x}{x} \right)^{1/2} \frac{d}{dx} \left[x^3 \left(\frac{1-x}{x} \right)^{1/2} \frac{dg}{dx} \right] - \frac{1}{2} g + \frac{x^3}{6} g^3 = 0 \quad (x > x_0) \quad (2)$$

since, as a consequence of the Einstein equations, $R \propto (p - (\rho/3))$. The vacuum energy of the Higgs field is minimised by the trivial solution, $g = \phi = 0$ for $x < x_0$. In order to find the stable vacuum for $x > x_0$, we notice that by introducing the variable $y = (1-x)^{1/2}$ equation (2) for $x \approx 1$ (near maximal expansion) becomes

$$\frac{d^2 g(y)}{dy^2} - 2[g(y) - \frac{1}{3} g(y)^3] = 0 \quad (3)$$

In analogy with the flat space case, the stable solution of equation (3) is found to be $|g| \approx \sqrt{3}$ ($y \approx 0$). Thus, the VEV of the neutral Higgs field is zero as long as the universe is "hot" ($x < x_0$) and then it grows gradually to the limiting value, $\phi_{\text{max}} = (\lambda a_{\text{max}}^2/3)^{-1/2}$ as the universe cools down ($x > x_0$). In this model, the value of λ can be adjusted to reproduce the presently observed ratio of weak and electromagnetic couplings.

Equation (2) can be integrated numerically for $x > x_0$. A typical solution (with $x_0 = 10^{-4}$) is plotted in Fig. 1. We found that the solution for $x \gtrsim 10^{-2}$ is very insensitive to the choice of x_0 within the above mentioned interval. Since $x_0 \ll 1$, the behaviour of $g(x)$ near x_0 can be estimated on the basis of the asymptotic solution near zero. There one finds $g(x) \sim x^\beta$ ($x \rightarrow 0^+$), $\beta = (\sqrt{(17-3)}/4) \approx 0.28$. Thus we approximate $\phi(x) \approx (x - x_0)^\beta$ near x_0 ; this behaviour is borne out by the numerical solution. Together with the relation between temperature and expansion parameter, $T x^2 \approx \text{constant}$, we thus estimate the critical exponent of ϕ to be $\beta \approx 0.28$ with a critical temperature $T_c \gtrsim 10^5$ K.

Since the effective weak interaction constant, G_F , is proportional¹ to ϕ^{-2} , its value changes very little during the present age: $\dot{G}_F/G_F = -2 \dot{x} d \log g/dx \lesssim -10^{-10} \text{ yr}^{-1}$. But it is remarkable that according to this model, weak and electromagnetic interactions were of comparable strength (and of long range) within the primordial fireball. The consequences of this result (particularly with regard to a neutrino background) are at present under investigation.

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A Megalithic observatory on Dartmoor

ASTRONOMICALLY aligned standing stones have been shown to occur at many sites dating from the late Neolithic and early Bronze Ages in North-west Europe. We have examined the stone rows at Merrivale on Dartmoor (SX553746) and conclude that they, together with associated stone rings and menhirs, form a solar and lunar observatory. An interesting feature of this site is that it apparently includes a calculating device for facilitating the prediction of lunar eclipses. Thom^{1,2} has suggested that certain stone settings which are themselves not astronomical alignments, notably four fan-shaped arrangements of stones in Caithness (Mid Clyth, Dirlot, Loch of Yarrows and Camster) and the well known groups at Carnac and Kermario, are in fact for this purpose. The Carnac alignments are complicated and difficult to interpret completely, the Caithness settings have been disturbed and many stones are missing; at Merrivale the stone rows have not been greatly damaged and the simple method of their use, though not identical to that proposed by Thom, lends support to his basic contention that techniques for lunar eclipse prediction were developed at that period.

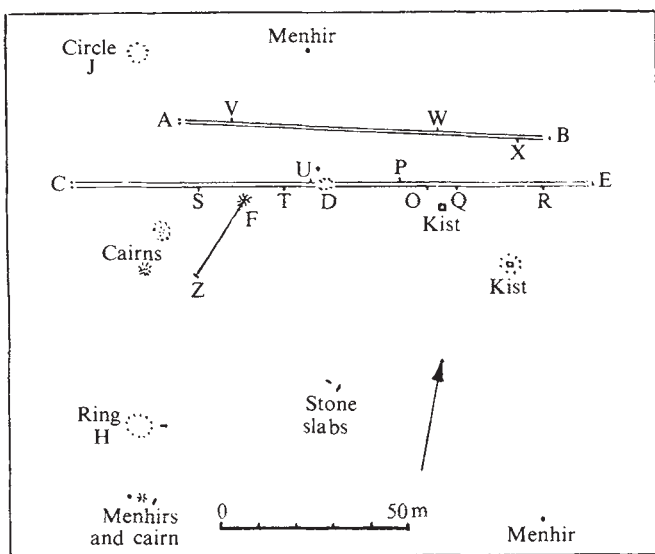


Fig. 1 The stone row site at Merrivale, Dartmoor.

We do not describe the Merrivale site in detail here, as it is hoped to publish a full account elsewhere. The stone rows (Fig. 1) lie on the summit of a fairly flat-topped bluff, which is covered with typical moorland vegetation of rough grass on peat and is, for the most part, free from boulders. There are two double rows, both roughly east-west; Row AB, 181.7 m long on an orientation of 83.7°, has 91 stones in its northern line, and 95 in its southern, most occurring in equally spaced pairs. Row CE has a length of 263.7 m, and is on an orientation of 81.7°. We found, with probing, 136 stones in the northern line and 130 in the southern. Neither double row is exactly straight, deviating about 2 m from the straight line, nor has a constant separation between the rows, which varies irregularly from 0.6 to 1.2 m. Both these rows have pairs of large stones to mark their western ends and a single stone set transversely to the rows at their eastern ends. Row AB has smaller stones than Row CE on average, but they are more uniformly spaced, with an average spacing of 1.86 m, whereas in Row CE the spacing changes from 2.05 m at the western end to 1.12 m at the eastern.

Row CE has a small egg-shaped ring, D, at about its mid point surrounding a cairn. Both rows have occasional large stones, (V, W, X, S, T, U, O, P, Q, R) along their lines, which stand about a metre high, compared with an average height of less than 0.5 m for the rest.

A third row of single stones, FZ 42.3 m, is at an angle of 57° to Row CE. The end of the row begins on the top of a small cairn and its first stone, F, is set with its broad face across the line of the row, as is the final stone Z. Very few stones are visible above the peat, and these are only about 0.1 m high. The row was probed and the peat was lifted to confirm the existence of possible stones. This row appears to be virtually complete, and there are 41 stones with a mean interval of 0.98 m. Apart from F and Z, none of the stones could have been more than 0.25 m above ground level, even in the Early Bronze Age, before the peat accumulation started.

About 100 m south of the rows is a well preserved ring H, of eleven stones (flattened circle, Thom Type B) and about 50 m north-west of A there is a true stone circle, J. The site also has several cairns, kists and four menhirs.

The site was surveyed by Lukis³ in 1879 and again by Worth⁴ who has also described some of its features⁵. During the investigation the site was resurveyed by sighting on the corners of three buildings whose 10-figure grid references were supplied by the Ordnance Survey Archaeological Division.

The north-west horizon, about 1.6 km from the site, is a smooth undulating moor with an elevation between 2.5° and 3.5°, broken by three separate outcrops of granite. Two of these outcrops make distinctive notches on the horizon. One of these, when viewed from the centre of ring H would have indicated the position of midsummer sunset (declination ϵ) in late Neolithic times, a second notch could have marked the position of the Moon, setting with declination $\epsilon + i$ (where i is the inclination of the Moon's orbit to the ecliptic) when viewed from the centre of J (Fig. 2).

We have been unable to find any outliers to the two stone circles which explicitly indicate these distant foresights. But two stone slabs in the centre of the site indicate the notch L on Fig. 2 and an outlier to the "egg" at D indicates notch M.

Although the horizon in other directions has prominent features, none of these is in a position to enable astronomical alignments to be made in other quadrants. Furthermore, the rows themselves are not aligned with astronomical significance.

For the present purposes the Moon's movements in declination may be regarded as a sinusoidal oscillation of amplitude ϵ and period 27.32 d, sinusoidally modulated by an oscillation of amplitude i and period equal to the rotation of the lunar nodes, 18.61 yr, and a minor perturbation, Δ , of amplitude 0.15° and period equal to half an eclipse year, that is 173.3 d. Whenever Δ is a maximum, the moon is at an "eclipse danger period". Thom points out that if Megalithic astronomers could make measurements that were accurate enough to observe the minor perturbation, they would be in a position to predict the possibility of lunar eclipses⁶. In essence this can be done by moving one's observation position from moonset to moonset, so that it seems always to set behind a suitable distant notch. Markers on the ground would effectively indicate a scale of declination measurement.

We propose the stone rows at Merrivale were used in this way, and that their purpose is to provide a permanent scale of measurement. The suggested method of use is that the observer moved, at each lunar setting, to that position along the double rows where he would see the Moon set in exactly the same place behind notch M. He could then place a temporary marker between the stone rows to indicate where he stood, and markers placed at successive moonsets would readily reveal the day on which the Moon had set with maximum declination, because on that occasion the markers would be nearest to the western ends of the double rows. The small deviations of the stone rows from straight lines would not materially affect the results. The rows are of such a length as to cover a lunar declination range of 26.0° to 30.1°, which means that they could have been used

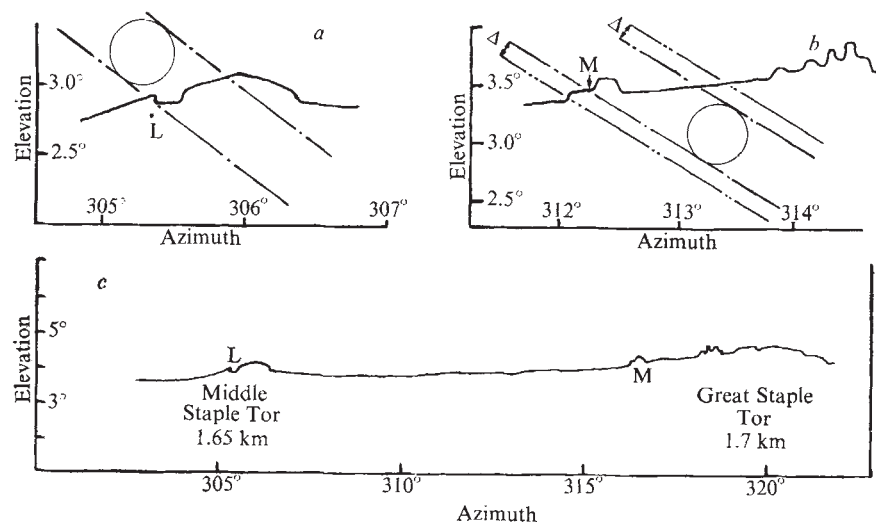


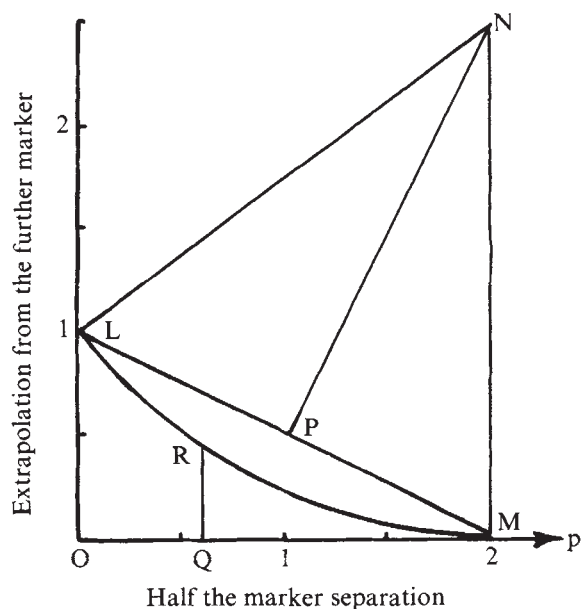
Fig. 2 *a*, The path of the setting Sun as seen from the centre of stone ring H in 2500 BC; *b*, the path of the setting Moon as seen from the centre of the stone circle J in 2500 BC; *c*, the north-west horizon as seen from the stone ring at Merrivale.

for about two years on either side of the declination maximum in the 18.61-yr cycle.

To achieve sufficient accuracy, the method needs to be elaborated, because the Moon may not be at its maximum monthly declination at the hour when it sets, and relatively small departures from the maximum would obscure the minor perturbation. Thom suggested a method by which the true monthly maximum declination could be obtained geometrically from the positions of markers placed on two successive nights. He shows that if the two markers nearest to the declination maximum are spaced a distance apart, $2p$, one must extrapolate a distance $G - p + (p^2/4G)$ from the further marker to find the position where one would have stood, if the Moon had set with maximum declination for the month. G is a constant for the site; it is the ground equivalent to half a day's change in declination from the maximum, and its value could have been found empirically by the prehistoric astronomers.

Thom's method involves the fan-shaped stone settings referred to earlier, and gives a precise value for the extrapolation distance $G - p + (p^2/4G)$, but our method is to approximate the parabola given by Thom's expression by the arc of a circle.

Fig. 3 The curve LRM represents the extrapolation distance $G - p + (p^2/4G)$ as a function of p . It is approximated by the arc of a circle centred at N with radius $NM = 2\frac{1}{2}G$. Scales in units of G .



Thus on Fig. 3 a circle centred at N with radius $2\frac{1}{2}G$ very closely matches the parabola LRM. If this construction were to be laid out on the ground, then to find the required extrapolation distance, one would lay off a distance MQ equal to half the marker separation, and the perpendicular, QR, from the baseline to the arc would be the extrapolation. This geometric construction is not the only solution; the same extrapolation distance is obtained if the circle has a radius of $8\frac{1}{2}G$ and the whole of the distance between the markers is laid off along MO. In general, if a fraction r of the marker separation is laid off, the correct value for the radius of the circle is $\frac{1}{2}(1 + 16r^2)G$. This method of extrapolation appears to have been followed at Merrivale using the short stone row FZ.

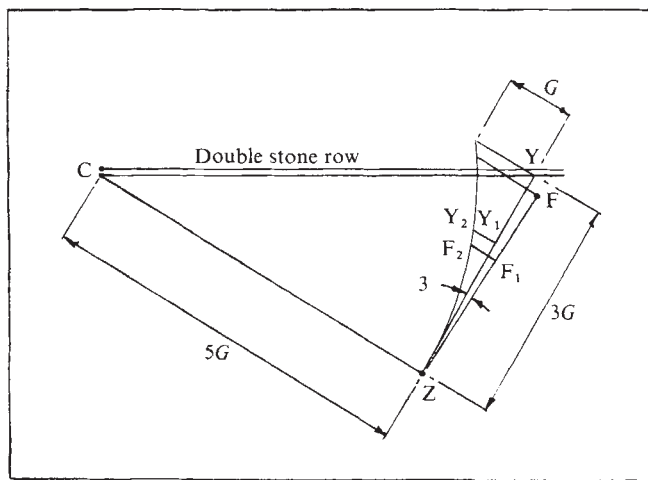


Fig. 4 The method of extrapolation at Merrivale. Lay off a distance FF_1 equal to $\frac{1}{2}\sqrt{2}$ of the marker separation; the extrapolation distance is the perpendicular F_1F_2 to the circle radius $5G$ centred at C. This gives a more accurate extrapolation than by laying off YY_1 equal to $\frac{3}{4}$ of the marker separation.

The Merrivale site has many interesting geometrical properties, including the repetition of certain units of length in its construction. One of these units is 15.6 m, which occurs as multiples along row CE in the spacings between the large stones. Thus we find (to within 1%) $OQ = 1$, $QR = 3$, $OR = 4$, $CU = 8$ and $UE = 9$ units. We are inclined to equate the unit of 15.6 m with Thom's constant G , which is calculated, using Heggie's method⁸, to have a mean value of 15.3 m over the site. The rows incorporate a triangle CZY (Fig. 4) in which ZY is the perpendicular to CZ, and if our interpretation is correct, two sides of the triangle, CZ (78.2 m) and ZY (45.6 m) are close to $5G$ and $3G$ respectively. This triangle ($r = \frac{3}{4}$) would therefore be one of the

family that could be used for extrapolation. The method would be to measure the distance between markers on successive evenings, lay off three-quarters of this distance from Y to Y_1 , and then project the perpendicular Y_1Y_2 to meet the circle centred at C. The length of this perpendicular is the distance to be added westwards from the further marker to get the position that the marker would have indicated, if the Moon had set with its monthly declination maximum.

In fact the short stone row FZ is not quite perpendicular to CZ and its length is 2.71G, not 3G. This slight modification to the simple scheme reduces the error inherent in this method of extrapolation, due to approximating the parabola with an arc of a circle, by about a factor of four and to a magnitude where it is insignificant. The distance to be laid out along FZ is not now $\frac{3}{4}$ of the marker separation, but $\frac{1}{2}\sqrt{2}$ which can easily be obtained by a simple geometrical construction using strings and pegs.

In examining prehistoric sites from an astronomical viewpoint one must guard against introducing fortuitous alignments or selective analysis of data. Thus one test for validity is the accuracy of the demonstrated alignments. Some authors, notably Thom, have taken great trouble with their surveys to find the exact declination of the Sun or Moon for the observing lines. In the present case it is difficult to apply this type of test, since the hypothesis is that the observers moved along the stone rows until they were observing the moonset exactly behind a particular distant feature. A test that can be applied is whether the hypothesis plausibly, and preferably quantitatively, explains the archaeological remains. The hypothesis advanced in this letter gives an explanation for the existence of the stone rows at Merrivale, including the precise dimensions and siting of the short single row, but there are many features of the site which we are unable to explain, of which the most obvious is why there are two double rows, for one apparently duplicates the function of the other. We have not examined any other site on Dartmoor.

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Palaeozoic secular variation recorded in Pendleside Limestone

RESULTS discussed here demonstrate the value of limestones in palaeomagnetic work. The fact that these rocks can apparently record secular variation provides evidence that the magnetisation is of depositional or very early diagenetic origin. Limestones may become a valuable tool in studying the ancient geomagnetic field.

Records of long term variations in the Earth's magnetic field over the past 15,000 yr or so come from lake sediments¹, marine sediments², archaeological materials³ and also from observatory records covering the past few hundred years.

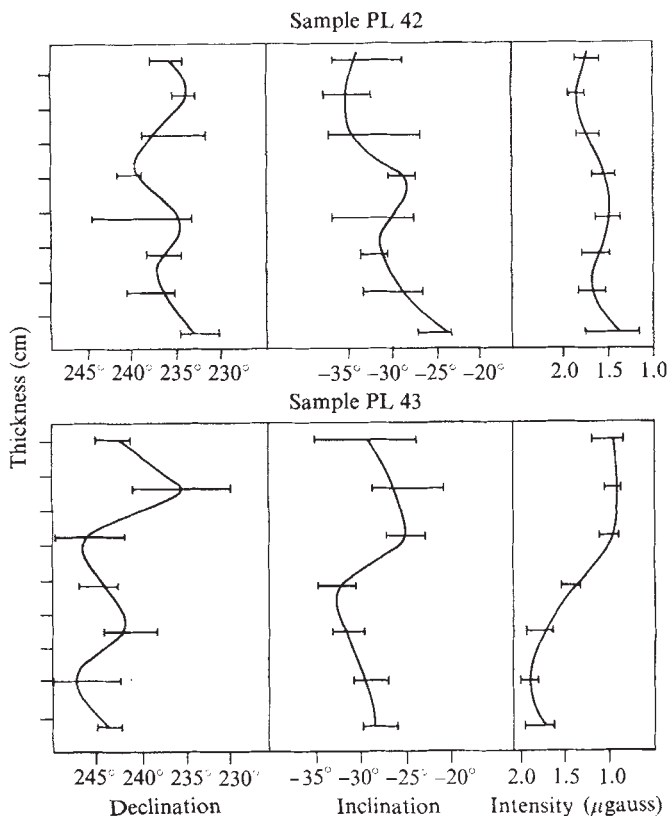


Fig. 1 The natural remanent magnetisation of two hand samples of Pendleside Limestone after a.c. cleaning at 150 oersted. Continuous curve is the mean of three curves obtained by slicing the cores into 1-cm disks. The error bars show the range of variation at each level.

Secular variations have also been convincingly demonstrated in Tertiary lavas⁴ and Mesozoic and Tertiary sediments. An important feature of secular variation is its strong dependence on latitude. Analyses of Recent geomagnetic data^{5,6} show that along lines of latitude progressively nearer the Equator, geomagnetic field directions become progressively more dispersed. Thus, the magnitude of secular variation should be greater nearer to the Equator. Tarling⁷, using the 1965 field data found a circular standard deviation ranging from about 7° near the poles to 19° near the Equator.

Although there have so far been no convincing records of Palaeozoic secular variation (before 225 Myr BP) variations could be preserved in certain Lower Carboniferous (Visean) limestones⁸. The available evidence suggests that the magnetisation of these limestones, which is highly stable and easily measurable, was acquired during or very shortly after deposition.⁹

To test this hypothesis a large palaeomagnetic collection of over 50 sites (including a few hand samples) has been made. None of the results from the collection has been discounted and all will be reported elsewhere. Results from two hand samples (44 specimens) are presented here as they provide a convincing demonstration of secular variation in Palaeozoic limestones. The sampled section is in the upper part of the Worston Shale Group and the Pendleside Limestone at Salterforth railway cutting (OS Ref. SD 889459) in northern England. It consists of alternating dark, fine grained limestones and shales. The two hand samples discussed here were collected from different horizons 11 m apart in the Pendleside Limestone. Three cores were drilled perpendicular to the bedding plane in each sample and then sliced into seven or eight 1-cm disks which were measured using a Digico magnetometer. The disks were then cleaned in an a.c. field of 150 oersted and remeasured

(Fig. 1). Several factors suggest that the results record Carboniferous secular variation.

- The variation in declination and inclination approximates to a sine wave and is quite regular. The amplitude of the variations is comparable with other secular variation records. In view of the low palaeolatitude of the Pendleside Limestone (15°) it may be expected that the amplitude of the variations should be greater. There are, however, two reasons why the amplitude of the variations is anomalously low. In the first place, the Carboniferous geomagnetic field may have been much steadier than the Recent field. In the second place, and this seems much more likely, the smoothing may have been brought about by each specimen acquiring its magnetisation over a long period of time. Only if the magnetisation were acquired penecontemporaneously would the full amplitude of the secular variation cycle be preserved. The degree of smoothing shown by the curves shows that the magnetisation of each specimen partly represents an integration of contemporary geomagnetic fluctuations. This supports the contention that the magnetisation of the limestones is of early diagenetic origin⁹.

- The periodicity of the variation in declination is compatible with Recent records, assuming a sedimentation rate of 1 cm every 500 yr; such a sedimentation rate is quite consistent with our present knowledge of marine carbonate sedimentation rates.

- The validity of the variations is supported by the correlation between the three cores in each hand sample. Both declination and inclination plots are all in close agreement and only one measurement, in the declination of PL42 (Fig. 1), is slightly out of phase.

- Comparison between the declination and inclination curves indicates that the variation in inclination is less than that in declination (Fig. 1). This is generally the case for many secular variation records. Moreover, both curves show trends with two declination swings to one inclination swing. This is another feature common to many secular variation records.

- There is some agreement between inclination and intensity of magnetisation, such as may be expected were the variations really secular variations. Low-field susceptibility has been measured to check if the intensity variation is a function of magnetic mineral content. This is not the case because no variation in susceptibility (13.5 μ gauss oersted⁻¹) could be detected within or between cores.

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Earliest calcareous foraminifera

WE present here evidence that early Palaeozoic calcareous foraminifera existed and were relatively complex (multilocular and multiserial) in form but that they have been regarded formerly as calcareous algae. The early part of the invertebrate fossil record during the Cambrian period seems to show a striking contrast between the abundance of highly developed organisms such as the trilobite arthropods and the paucity of simpler forms such as the foraminiferal protozoans. Foraminifera are very small acellular animals with an important role in marine food chains¹ and it is reasonable to expect

them to have been a significant component of the benthic fauna throughout the Palaeozoic. Yet few foraminifera have been reported from the Lower Palaeozoic and those described from the Cambrian are mainly simple forms with unmineralised tectinous or agglutinated tests^{2–4}. Some more complex agglutinated forms are known from the Upper Cambrian⁵, but the first generally recognised calcareous foraminifer is the uniserial *Saccaminopsis* from the late Ordovician⁴. Consequently, the assumptions that Lower Palaeozoic foraminifera were primitive and mainly non-calcareous have been taken as bases for inferences concerning the early evolution of the group^{3,4} and the paradoxical, yet apparently inescapable, conclusion has been drawn that Cambrian foraminifera are very scarce.

We describe here small, septate chambered organisms with a thick calcareous wall belonging to a generic group centred on *Renalcis* Vologdin, and termed here renalcids. At least six nominal genera are involved (*Renalcis*, *Nubecularites* Maslov, *Chabakovia* Vologdin, *Shuguria* Antropov, *Izhella* Antropov, *Nephelostroma* Dangeard and Doré) although only two, *Renalcis* and *Izhella*, are likely to prove acceptable. Renalcids range throughout the Cambrian and are also well known from the Lower Ordovician and Upper Devonian. *Renalcis* has also been found recently in the Silurian of Khazakhstan, USSR (I. T. Zhuravleva, personal communication). They have a global distribution and are locally very abundant in a variety of early and middle Palaeozoic bioherms constructed by stromatolites, archaeocyathids, sponges and stromatoporoids⁶. Various affinities, some foraminiferal but mostly algal (Table 1), have been attributed to renalcids. The view that they are algae is not consistent with their morphology and a more satisfactory explanation is that they belong to the Palaeozoic foraminiferal superfamily Parathuramminacea⁷.

Renalcids have a microgranular calcareous wall and a septate chambered structure. An individual test can include from 2 to more than 80 chambers. Internal chamber sizes range from 10×40 to 200×250 μ m. Wall thickness ranges from 5–125 μ m. The chambers are connected by intercameral foramina in the septa (Fig. 1). Apertures seem to be lateral and there are no pores in the wall. The complete test is generally between 500 μ m and 1 mm in size. These dimensions are based on numerous measurements of Cambrian and Devonian specimens. Chambers are usually arranged in several branching series. In random thin sections these seem to arise from a cluster of small chambers (Fig. 1) or from a large initial chamber which may be the proloculus. The specimen shown in Fig. 1 is from the Devonian and has been selected for its good state of preservation, but closely comparable specimens have been figured from the Cambrian of Siberia⁸. Renalcids may occur free in fine-grained sediment or attached to larger skeletons⁹.

Attributions of algal affinity to renalcids have been conflicting and are difficult to support. Both blue-green and red algae have been referred to. Blue-green algae are either filamentous or coccoid in form. Calcified filamentous blue-greens produce non-septate tubiform skeletons ascribed to genera such as *Girvanella* and *Ortonella*⁹. These are much smaller and totally

Table 1 Affinities formerly attributed to *Renalcis*, *Izhella* and their synonyms

	Blue-green alga	Red alga	Foraminifer
<i>Renalcis</i>	Korde ⁸ Johnson ²¹ Wray ²⁵	Korde ¹¹ Vologdin ²⁶	
<i>Nubecularites</i> <i>Chabakovia</i>	Maslov ¹⁰ Voronova ²⁷	Korde ⁸ Vologdin ²⁶	Elias ¹³ Klován ¹⁴ Antropov ¹⁵ Loeblich and Tappan ⁴
<i>Shuguria</i>			
<i>Izhella</i> <i>Nephelostroma</i>	Antropov ²⁸ Dangeard and Doré ²⁹		

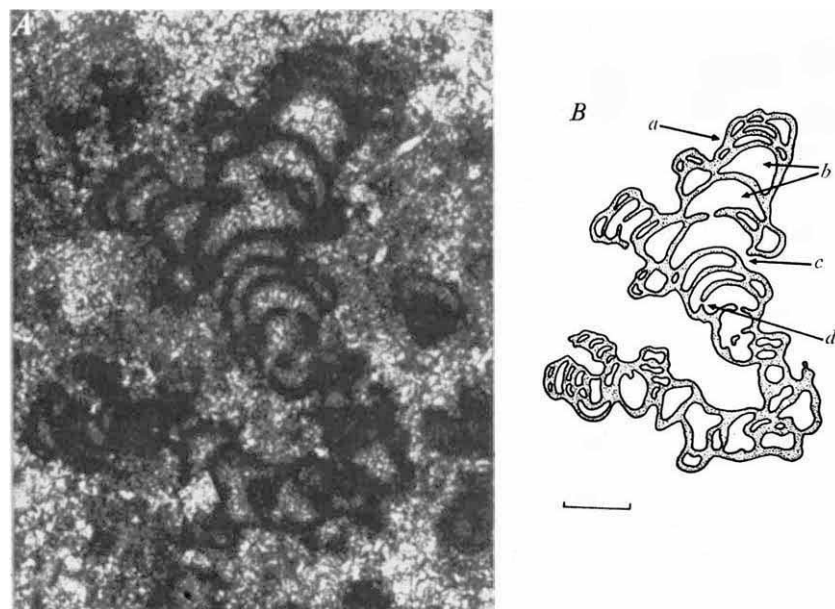


Fig. 1 Test of *Renalcis* sp. (Upper Devonian, Mount Hawk Fm., Mount Haultain, Alberta, Canada), showing branched multilocular form. A, Photomicrograph ($\times 50$); B, diagram indicating foraminiferal features: a, microgranular calcareous wall; b, chambers; c, lateral aperture; d, intercameral foramen; scale bar, 250 μm .

different in morphology from renalcids. Calcified coccoid blue-greens are not known from the Recent but calcification of the mucilaginous sheath surrounding coccoid cells could also be expected to produce skeletons morphologically and dimensionally quite distinct from renalcids. It is inconceivable that the chambers of renalcids represent individual blue-green cells, as Maslov¹⁰ has suggested, since their sizes differ by an order of magnitude. Korde^{8,11} suggested that the renalcid wall has a minutely cellular structure which indicates an affinity with red algae. But the cellules she reported are orders of magnitude smaller than the cells of undoubted fossil and Recent calcareous red algae. Further, we have been unable to detect cellular microstructure in the walls of renalcids from a variety of geological situations. Rather, the tests seem to comprise finely granular calcite with no visible structure, an observation supported by scanning electron microscope (SEM) studies¹².

The size and morphology of renalcids are, however, consistent with their being foraminifera. The skeletons consist of chambers, connected by intercameral foramina and with lateral apertures, arranged in extended branching series. A foraminiferal affinity for renalcids has received some previous support^{13,14} (Table 1). Even so, uncertainty has remained concerning their precise position. Elias¹³ thought that *Chabakovia* was related to *Ptychocladia* and placed both in a new foraminiferal family, Ptychocladidae. Loeblich and Tappan⁴ placed this family in the Endothyraea, but tentatively removed *Chabakovia* to the problematic group Reitlingerellida. They placed *Shuguria* in the parathuramminacean family Caligellidae. In our opinion the rather irregular form, relative morphological simplicity, and finely granular unlayered calcareous wall favour the placement of the renalcids as a discrete group in the Parathuramminacea⁷.

We propose to name this family Renalcidae, defined as follows. Test free or attached, irregular, branching uniserially; chambers numerous, lunulate to hemispherical, early chambers inflated; wall thick, calcareous, microgranular; apertures lateral. Type-genus *Renalcis* Vologdin. The Renalcidae differ from other parathuramminacean families in having thick walls, closely compressed chambers, and a tendency to branch. They most closely resemble the Caligellidae but differ in having a well developed septation, more compact form, and a lack of well defined coiling at any stage. The Ptychocladidae are very similar to the Renalcidae in the form of their branched uniserial test, but differ in their prostrate and always attached habit, radial partitions in the larger chambers, and layered wall.

The foraminiferal nature of renalcids has significant bearing on the early history of the Foraminifera: calcareous stocks now seem to be as ancient as agglutinated stocks, provided

that dubious Proterozoic specimens⁵ are disregarded. *Renalcis jacuticus* is one of the first calcareous organisms to appear in the lowest stage of the Cambrian, and the underlying Precambrian Yudomian strata have yielded *Nubecularites abustus*¹⁶.

In the Baltic region the agglutinated foraminifer *Platysolenites antiquissimus* (homeomorphic with *Bathysiphon*) is, likewise, one of the first shelled organisms to appear in the sequence, occurring in the Precambrian Vendian strata¹⁷. The Vendian and Yudomian are of similar age¹⁸ and the detailed biostratigraphy is uncertain, so that it is hard to say precisely which stock, calcareous or agglutinated, is the older. Consequently, it is difficult to support the inference that all calcareous foraminifera arose from agglutinated stocks as suggested by Loeblich and Tappan¹⁹. Both seem more likely to have arisen independently from simple, late Precambrian, tectinous forms.

With the recognition of renalcids as foraminifera two Cambrian foraminiferal biofacies now become apparent. Agglutinated microfaunas (for example, *Scaniella*, *Platysolenites*, *Psammospaera*, and *Hippocrepina*) dwelt in predominantly argillaceous sediments, associated with hyolithids, hyolithellids, trilobites, brachiopods, and siliceous sponges²⁰. Conditions were probably turbid and cool^{17,20}. Calcareous microfaunas (renalcids) dwelt in predominantly calcareous sediments associated with archaeocyathids, trilobites, chancellorids, gastropods, and the alga *Epiphyton*. Conditions were probably warm, clear and shallow²⁰. Warm, shallow-marine, carbonate environments of Upper Palaeozoic–Recent age also contain abundant calcareous foraminiferal assemblages. If renalcids were lacking it would be difficult to explain why similar habitats were unoccupied by calcareous foraminifera in the Lower Palaeozoic, especially as conditions favoured many other shelled organisms, and other not entirely hospitable environments supported foraminiferal assemblages.

The basic features of the renalcid test may be explained ecologically. Warm, CaCO_3 -saturated waters favour the secretion of thick calcareous walls, whereas agglutination is favoured in cooler or less saturated waters²¹. Chambers and septa, formed by periodic growth, serve as physical and osmotic barriers for the protoplasm inside²². Protection of this kind is of value in shallow-water biohermal environments where temperature, water-movement, predation, and ultraviolet radiation are likely to be high and where plant respiration and photosynthesis may cause local fluctuations in pH and Eh. Chambers may also help to culture algal endosymbionts, an association commonly found in shallow-marine tropical foraminifera²³.

We conclude that renalcids were the earliest calcareous foraminifera and belong to the superfamily Parathuraminacea. Their presence in the early Cambrian suggests that calcareous foraminifera arose, independently of agglutinated foraminifera, from tectinous forms. Renalcids were adapted to the warm, shallow-marine, biohermal carbonate environments found widely across the continental shelves in early Palaeozoic times.

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Mechanism of oxidation of polyacrylonitrile fibres

The first and most important stage of the preparation of high modulus carbon fibres from polyacrylonitrile (PAN) textile fibres is the oxidation of the fibres under tension. This produces an oxidised ladder polymer structure approximately parallel to the fibre axis which may be regarded as the template for the formation of the oriented carbon fibre. The exact structure of this oxidised polymer is not clear although four different ones have been proposed^{1–4}.

The structure of the unoxidised ladder polymer, which is formed without weight loss by heating PAN in a vacuum at temperatures of 180–230 °C is well established however, and, neglecting initiation points, is a condensed naphthyridine ring structure⁵:

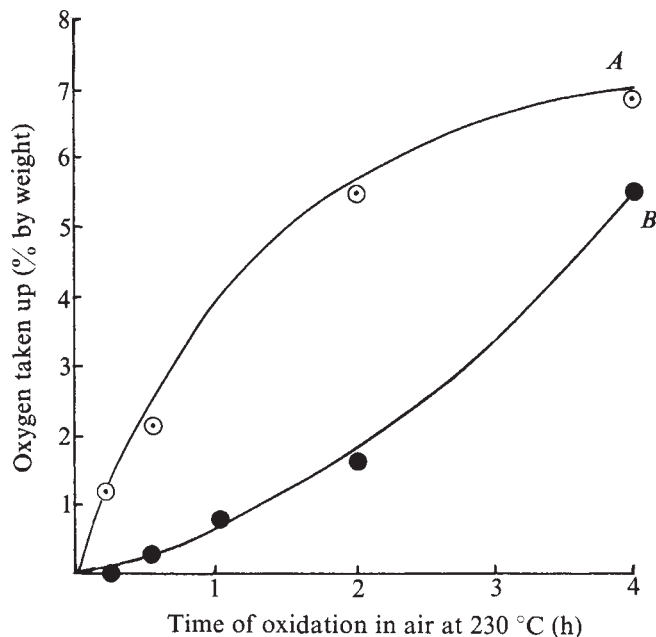
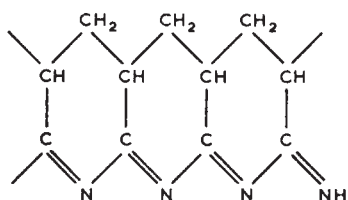


Fig. 1 Oxidation with no previous treatment. The values for oxygen taken up are the total found by analysis less the value before oxidation. A, Fibre A; B, fibre B.

This structure confers a copper colour to the fibres heated in vacuum because of the conjugated $-C=N-C=N-$ system.

Textile PAN fibres are copolymers of acrylonitrile with one or more comonomers which are added to provide dye sites and also, by introducing points of structural inhomogeneity, to act as internal plasticisers and make fibre drawing easier. The various proprietary PAN fibres have different comonomers in them, a common quantity being about 6% by weight. It has not yet been shown, however, what effect these different comonomers have on the oxidation kinetics of PAN fibres. In this communication we compare the oxidation kinetics of two commercially available PAN fibres.

Fibre A was a round wet-spun fibre of 1.5 denier (cross-sectional area $133 \mu m^2$) of composition 95.0, 4.6 and 0.4 mol % of acrylonitrile, methyl acrylate, and a vinyl acidic compound providing $-COOH$ groups directly attached to the all-carbon polymer chain. Fibre B was dry spun with a 'dog-bone' cross section of 1.8 denier (cross-sectional area $165 \mu m^2$) and of composition 95.4 and 4.6 mol % of acrylonitrile and methyl acrylate, respectively.

The oxidation of the fibres was investigated by estimation of the oxygen absorbed after different oxidation times at 230 °C in air with the fibres tied on a glass frame and by examination with an optical microscope of cross sections of oxidised fibres cut to $1 \mu m$ thickness on an LKB microtome. The oxygen absorbed is shown in Fig. 1 and the cross sections of the fibres after 4 h of oxidation are shown in Fig. 2A and B. Fibre A has a fast initial oxidation rate and the sections showed what we have previously called an oxidation zone⁶ inasmuch as it moves inwards at a rate proportional to the square root of the oxidation time. Fibre B has a slow initial rate which increases with time, but at 230 °C does not show any oxidation zone, at least up to times of 4 h. The colour gradually changes uniformly over the section to a mid-brown.

As-received fibres were heat treated in a vacuum for 6 h at 230 °C, again while tied on glass frames. The weight changes were negligible but both groups of fibres were copper coloured, indicating extensive transformation into ladder polymer. The oxidation at 230 °C and microscopical examination of these fibres was carried out as before and results are shown in Fig. 2C and D and Fig. 3; the sections showed oxidation zones after only 1 h of oxidation. Clearly the vacuum heat treatment before oxidation has not changed the oxidation

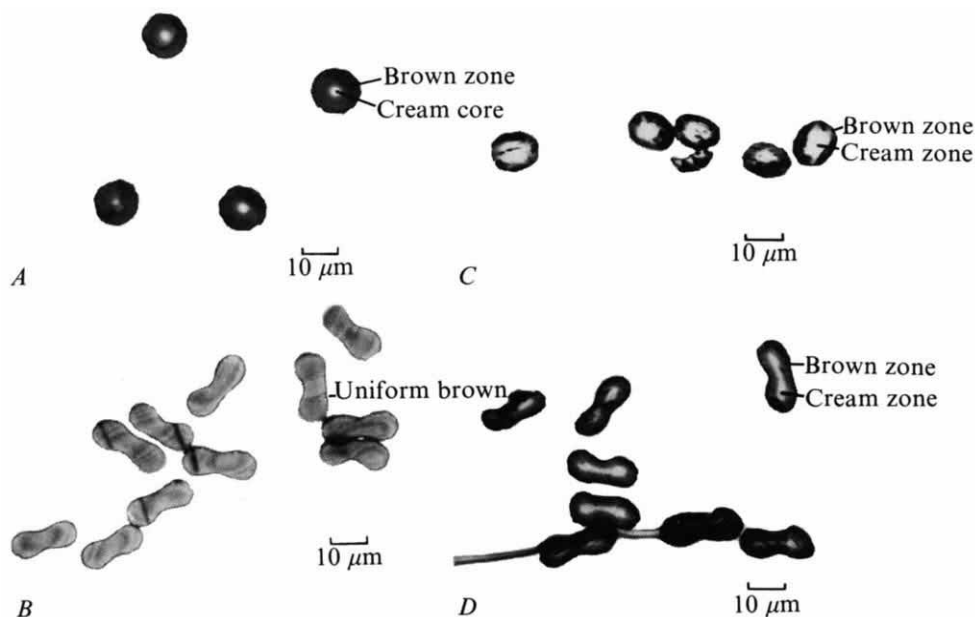
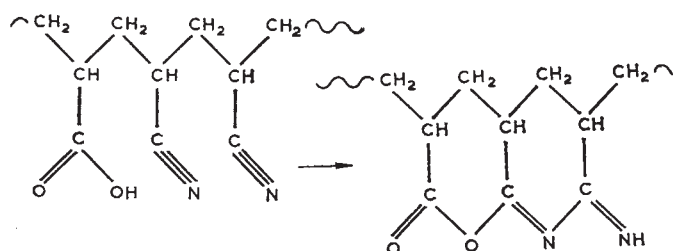


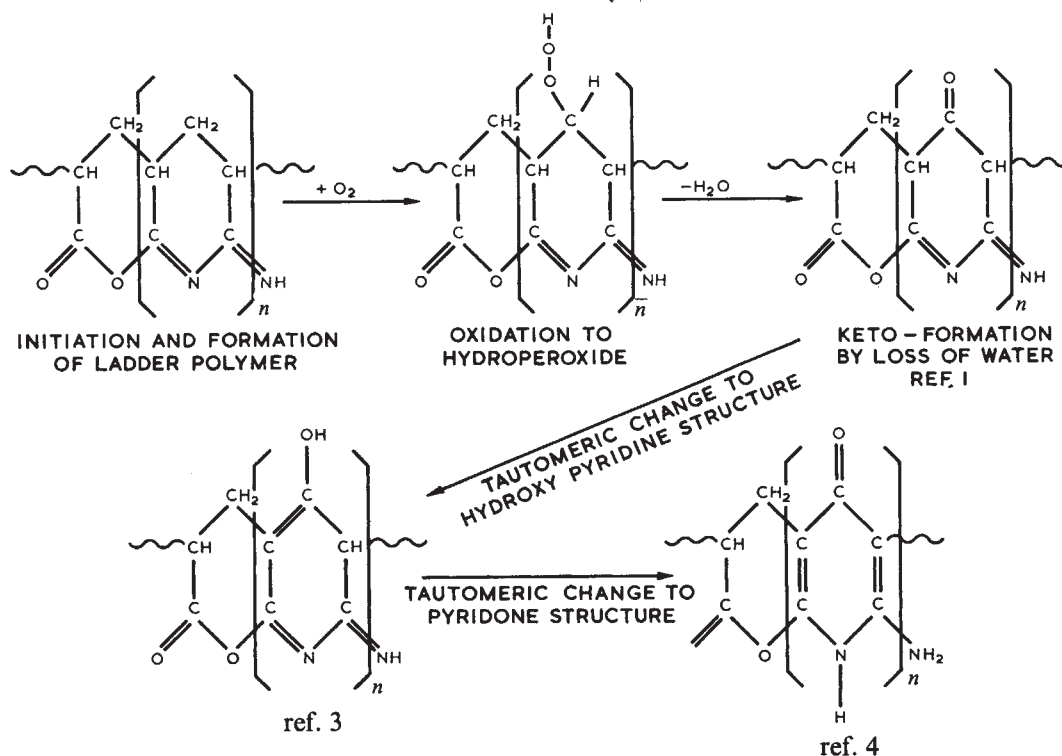
Fig. 2 A, B, Cross sections of fibres A and B, respectively, after 4 h in air at 230 °C. C, D, Cross sections of fibres A and B, respectively after 6 h in vacuum at 230 °C and 1 h in air at 230 °C.

kinetics of fibre A but has had a marked effect on fibre B, so that the oxidation kinetics are now similar to those of fibre A.

It seems that it is ladder polymer which is oxidised and this must be formed first. The acidic constituent of fibre A acts as an initiator for ladder polymer by the following mechanism as shown by Grassie, and this occurs rapidly at 230 °C:



Fibre B, with no acidic constituent, does not initiate ladder polymer formation very readily and this explains the different oxidation curves before and after the heat treatment. The presence of oxidation zones in fibre A, with or without heat treatment, shows that the rate of oxidation of the ladder polymer is faster than the rate of diffusion of oxygen in the fibre at 230 °C, whereas in untreated fibre B the rate of formation of ladder polymer and thus the rate of oxidation, is slower and cannot keep up with the rate of diffusion at 230 °C so that no oxidation zones can be seen unless the fibre is vacuum heat treated first. The conclusion drawn from these results, that formation of ladder polymer is a prerequisite to oxidation, is consistent with the structural formula for the oxidised ladder proposed by Potter and Scott⁴, based on the susceptibility to air oxidation of the ladder structure to give a pyridone structure. Three out of the four formulae proposed for the oxidised ladder polymer can, however, be reconciled if account is taken of oxidation mechanism and successive tautomeric changes, as follows:



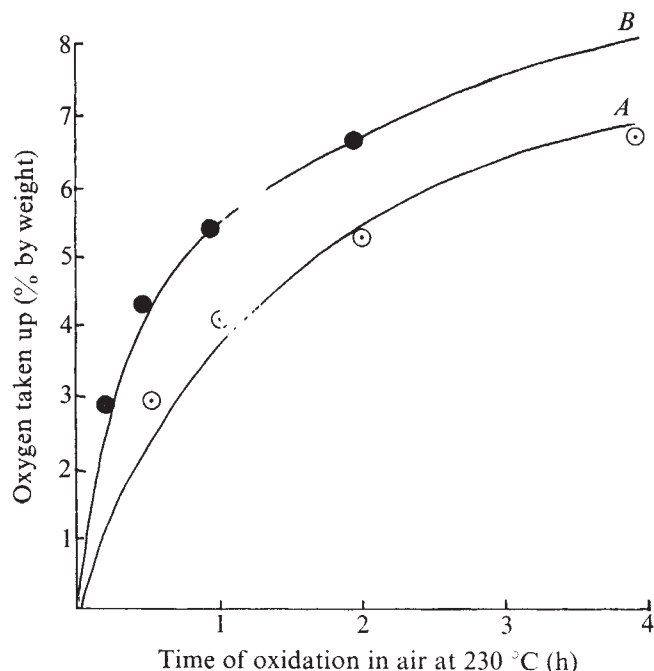


Fig. 3 Oxidation after 6 h of vacuum treatment at 230 °C. ○, Fibre A; ●, fibre B.

The lengths (n) of the conjugated sequences are probably about 4–5 monomer units and the formulae proposed apply only to the oxidised ladder polymer. No account has been taken of some chain scission which does occur during oxidation, as shown by the evolution of CO_2 (presumably from $-\text{COOH}$ groups) in the early stages of the subsequent inert pyrolysis to carbon fibres. These can be formed by oxidation of the polymer ends resulting from the scission, and these $-\text{COOH}$ groups thus formed can then initiate chain scission⁷. This may be the mode of initiation with fibre B, thus accounting for the acceleration of the oxidation rate with time.

The technique of examination of thin sections of oxidised fibres seems to be a new and sensitive technique of assessing PAN fibre oxidation kinetics and is being followed up in more detail. It is more positive than the examination of polished cross sections which we have previously used to investigate the oxidation of 3 denier ($284 \mu\text{m}^2$) PAN fibres⁶.

Our assumption that the outer zone was oxidised PAN whereas the inner core was not has been verified using an electron microprobe analyser tuned for oxygen detection and comparing the oxygen level in the zone and core of an oxidised fibre. It was found that the brown outer zone is relatively rich in oxygen compared with the light yellow core⁸.

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Artificially triggered lightning above land

LIGHTNING has been triggered artificially in the Massif Central by launching small rockets carrying a thin steel wire connected to the ground. During two investigations in 1973 and 1974, a total of 20 triggerings have been achieved. Photographic evidence establishes the existence of two kinds of luminous object, lasting several tenths of a second, which may be similar to some varieties of ball lightning.

A sudden perturbation of the electric field can trigger lightning^{1,2} and deliberate triggering of lightning with low cost equipment was first demonstrated by Newman *et al.*³, who launched small rockets carrying a thin wire connected to earth towards electrified clouds. The experiments were performed from a ship; our experiments extend this work to a fixed land-based station. Notwithstanding the lack of mobility, such a station does allow experiments that would be impossible or at least very difficult to perform at sea.

Our programme is the result of a joint effort by the Commissariat à l'Énergie Atomique (CEA) and Electricité de France (EDF). The wire and rocket technique was developed initially at the CEA in the hope of perhaps shedding new light on the controversial subject of ball lightning⁴, since according to published statistics⁵ ball lightning is mentioned in more than 40% of the cases in which an observer describes a nearby lightning stroke.

The experimental lightning station was installed by EDF⁶ at Saint Privat d'Allier in the Massif Central, a region where the isokeraunic (thunderstorm) level is about 32. The area is rather flat locally, at an altitude of 1,100 m in the vicinity of smooth mountains which reach 1,300 m. The station consists of three main parts: the staff shelter, which contains the control desk and various cameras; the EDF tower; and the CEA range. An observation hut has been installed at a distance of 2.7 km from the main complex.

The EDF metal tower is 24 m high and is 110 m from the shelter. During the 1973 investigation, rockets were launched from a platform situated half way up the tower under a copper ring, 4 m in diameter, fixed to the top. It was expected that the metal wire carried through the ring by the rocket would make an electrical contact sufficient to conduct the lightning current to the top by way of the ring. All lightning, except one, however, struck the platform directly at the first stroke. When the flash comprised several strokes the subsequent ones were captured by the ring. Consequently, for the 1974 investigation the tower was modified for launching the rockets from its top. Four launchers were permanently loaded on the platform in 1973 and six on the top in 1974.

Fig. 1 Triggered lightning photographed from a distance of 2.7 km.



The CEA range was installed to provoke the interaction of lightning strokes and of lightning current with natural materials. The underlying idea is that some organic or powdery material is present in many instances when ball lightning occurs. This has been noticed already by some ball lightning theorists. So in our experiments lightning was also attracted to the ground in a meadow where two launchers were installed in 1973 and four launchers in 1974. First, experiments were performed with the wire reel laid on the ground. Later it was placed on a porcelain insulating pillar about 1 m high. This was tried in an attempt to delay fusion of the wire and to get bigger strokes. Finally, during the 1974 investigation, each reel was fixed to the top of a vertical pipe 1.5 m high and 14 cm in diameter, made of asbestos concrete and internally coated with soot. This tentative disposition was adopted to try to reproduce the creation of ball lightning when a chimney is struck by lightning. Measurement of peak lightning current is performed with magnetic link arrays, nine links being placed on wooden supports with threefold symmetry around each pipe.

The station is equipped with several devices for storm detection and electric field measurement^{7,8}. During storms the decision to fire a rocket depends on the indication of a field mill electrometer. We used standard anti-hail rockets (Ruggieri type 614) attached to a 0.2 mm steel wire unwinding off a reel. The reel is supplied with a cylindrical metal casing fitted with a conical nozzle and brake; it is sufficiently small ($h = 20$ cm, outer diameter = 5 cm) for easy fixing at the place where one wants the lightning to strike. The electrically fired rocket pulls the wire to a height of 700 m in about 5 s. Then it is automatically destroyed in accordance with safety regulations.

Between mid-July and the end of September 1973, 12 triggerings were obtained with 20 technically correct launchings. In 1974, eight triggerings resulted from 10 correct launchings between June 1 and October 15. Eleven flashes out of a total of 20 had at least one intensity peak greater than 2 kA, the highest value being 19 kA.

Triggered lightning seems to be similar to upward natural lightning striking tall structures^{9,10}. As a rule, triggered lightning begins with a slow discharge, the intensity of which remains in the kiloampere range, or less, with a rise time longer than 10 μ s and a total duration of a few tenths of a second. In nine cases the slow discharge was followed by several restrikes with steep-front, high current peaks of short duration.

In the case of negatively charged clouds, pictures taken from the distant observation hut show that branching may be very important (Fig. 1). On this photograph the vertical visible extension is of about 2 km and the total lateral extension at least 4 km. The overexposed part at the bottom corresponds to the 150-m length of vaporised wire. The shape of the branches is characteristic of upward discharges. This is confirmed by cine film (48 frames s^{-1}) which shows a rather low upward speed of about 2×10^4 m s^{-1} at the beginning. Subsequent strokes are correlated with the successive illumination of various branches in which the propagation is too fast for our measurements.

With positively charged clouds, triggering seems more difficult in the sense that the field at the ground has to be higher for the same probability of success. In this case the discharge also propagates upwards but with a greater speed of the order of 10^5 m s^{-1} or more; branches are also less numerous. Of course these conclusions are tentative since they are based on a small number of events.

We have not observed anything comparable with the more remarkable tales of ball lightning folklore. Nevertheless our search for ball lightning has not been fruitless. Several observations have been made which have a connection with the subject and which may help to explain some occurrences of ball lightning.

The beaded appearance of triggered lightning during its decay was noticed when triggering was unexpectedly produced by a plume of water ejected by a submarine explosion¹. In our experiments, the beaded decay has shown up systematically, and increasingly with increasing lightning strength. It has been

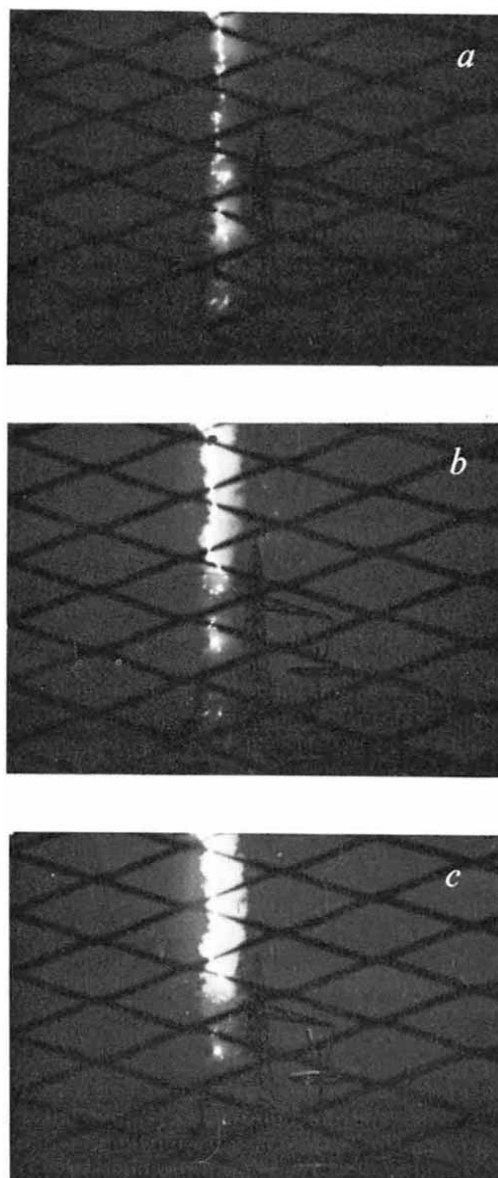
registered on pictures taken mostly with a camera (16-mm, 150 frames s^{-1}) at a distance ranging from 70 to 110 m, and with focal lengths from 30 mm to 120 mm.

The beads generally have an initial diameter of the order of 40 μ m which decreases gradually with a total lifetime of 0.3 s at most.

During long-lasting strokes, the initially straight channel adopts on a progressively more tortuous shape and the biggest beads occur where tortuousness is a maximum. Since there is a positive correlation between diameter and lifetime, it follows that at the end of the decay there are one or two luminous balls only. In general these objects have an upward motion of 1 or 2 m s^{-1} , which gives an overall picture consistent with the hypothesis of a gradually cooling spheroid of hot gas¹¹.

Moreover, it seems unjustified to invoke a persistent current to explain the long lifetime¹². This results from the following observation: on one occasion, after a stroke lasting 0.66 s on the EDF tower, a luminous spheroid was floating half way up

Fig. 2 A stroke on the tower. Pictures taken with a 16-mm cine camera at 100 frames s^{-1} . *a*, At $t = 0.66$ s after initiation of a stroke reaching the platform the "beads" in the decaying channel are irregular, as is generally the case after powerful strokes of long duration; *b*, at $t = 0.68$ s a restrike occurs, illuminating the smoke trail left by the rocket, and stops on the upper structure of the tower leaving undisturbed the luminous remains below; *c*, at $t = 0.70$ s the process continues.



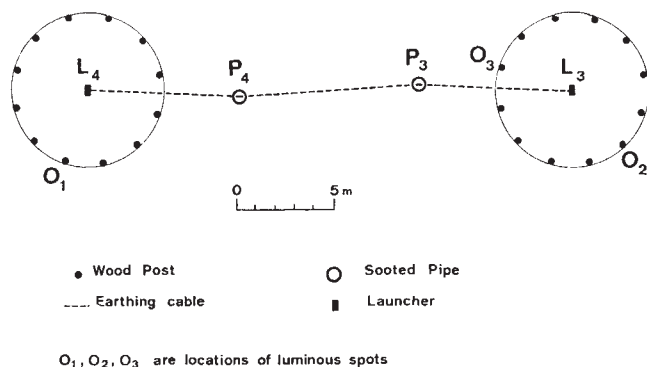


Fig. 3 Layout of a pair of launchers on the ground range and location of luminous spots.

between the launching platform and the metallic ring, then a subsequent brief stroke fell on the ring without disturbing the slow decay of the luminous object below. It is evident that, in the case of a decay governed by the input of an external current, a modification would have resulted from the change in electric field associated with the second stroke.

It can be stated also that the residue of the vaporised wire has little influence on the appearance and the behaviour of the beads. This is deduced from the following sequence of events:

In most cases the current in the first strike rises very slowly. The wire begins to break at the point where it leaves the reel, where the mechanical stress is a maximum. Then a straight arc is drawn upwards between the immobile reel and the lower end of the ascending wire. Complete vaporisation of the wire occurs only when the arc has reached a length of a few metres (typically 5 m). Then, as long as the current flows, the lower few metres emit much less light than the upper part, which is contaminated by metal vapour, but the difference disappears early during the afterglow. The similarity of the beaded decay in the lower and upper sections is indicative of the lack of influence of condensed wire residue. Incidentally, it proves also that the temperature of the beads must be rather low.

Photographic records reveal that during flashes striking pipes 3 and 4, light emission occurred at the foot of wooden posts supporting a wood fence erected for security reasons around each launcher. The general layout is shown in Fig. 3 which shows two launchers (L₃, L₄), two sooted pipes (P₃, P₄), the fences with the posts around L₃, L₄, the earthing cable (EC) and three places (O₁, O₂, O₃) where light emission has been noticed. For practical reasons the records on O₁ are more numerous and have a better quality than those concerning the two others. Since the few records on O₂ and O₃ bear much similarity with O₁ the following discussion will refer to O₁ only.

Photographic images have been obtained at a distance of 67 m with a 16-mm cine camera (150 frames s⁻¹, $f = 40$ mm) operated by us, a still camera ($f = 80$ mm) and another 16-mm cine camera (66 frames s⁻¹, $f = 10$ mm) operated by F. Ruhling (Institut für Hochspannungs und Anlagentechnik, Munich). Light from O₁ has been observed twice: first during a flash on P₄ with a maximum negative current of 2.8 kA and 12 min later during a flash on P₃ with a maximum negative current of 15.3 kA. The flash on P₄ lasted 0.55 s with fluctuations but without a sharp restrike. In that case, the light from O₁ appeared at $t = 0.23$ s after breakdown, grew progressively for 0.06 s, fluctuated in phase with the flash and disappeared at $t = 0.55$ s when the main channel began to break into beads. The flash on P₃ had a total duration of 0.8 s. It began with a discharge lasting 0.42 s and ended with eight restrikes. Light from O₁ appeared at $t = 0.21$ s after breakdown, ceased at $t = 0.44$ s and appeared again with each restrike until $t = 0.8$ s.

The light-emitting region was stationary, in contact with the ground at the place where the post enters the earth. Its shape seems roughly spherical with a diameter of about 25 cm but

because of the poor resolution these observations are somewhat tenuous.

The hypothesis of an accidental reflection of light from the flash is unfounded because in both cases the object appears later than the time of maximum brightness for the main channel. Visual inspection of the spot did not reveal anything special. No marks or burns were visible on the grass or on the post.

The most probable explanation is that it was due to the influence of electric currents circulating in the ground during the flash. These currents start from the earthing cable connecting L₃, P₃, P₄, L₄, which is buried only a few centimetres deep. It is not surprising that some underground outgassing occurs and that the gases escape at the point where the posts puncture the upper layer. It remains to be decided whether the light comes from hot gases only or from a combustion involving hydrogen or methane, for instance, or from a localised electric discharge mechanism. The fact that O₁ was pulsating in synchrony with the restrikes does not support the combustion hypothesis. Further experiments are planned to clarify the question.

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Isoelectric point of the edge surface of kaolinite

THE nature of the particle-particle interactions in clay suspensions is of great technological importance since these interactions determine the flow properties of suspensions used in, for example, ceramic manufacture and paper making, and as oil drilling fluids. They also influence profoundly the structure and behaviour of soils. Clay particles are of a plate-like morphology and thus exhibit two types of surface, the edges and the basal faces. Consequently, a variety of different types of particle-particle interaction are possible in aqueous clay suspensions^{1,2}. For example, structures consisting of individual particles coagulated in an edge-face or an edge-edge arrangement have been proposed as well as face-face (or 'card-pack') aggregates which may themselves be coagulated in edge-face or edge-edge modes³.

The type of structure present in a clay suspension is determined by the electrical double layers at the two interfaces. The results presented below are a preliminary report of a study designed to evaluate the influence of electrical double layers on the properties of aqueous kaolinite suspensions³ and hence to elucidate the conditions in which the

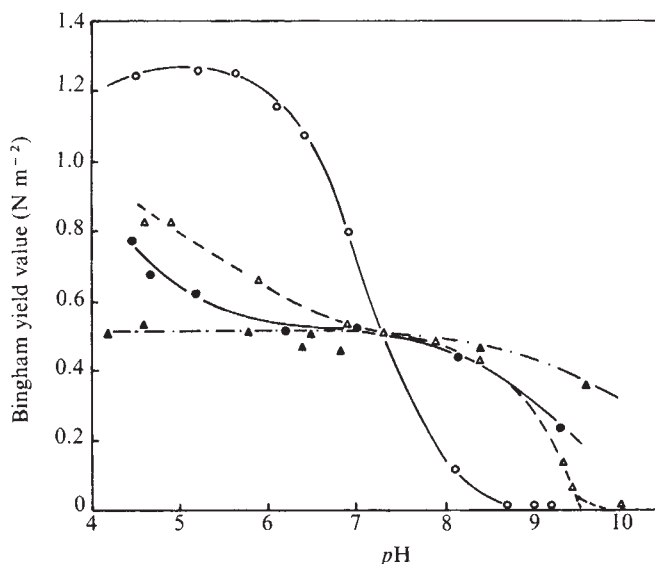


Fig. 1 Bingham yield values of homoionic sodium kaolinite as a function of pH and NaCl concentration. $\circ$, 10^{-4} M NaCl; $\triangle$, 0.017 M NaCl; $\bullet$, 0.034 M NaCl; $\blacktriangle$, 0.12 M NaCl.

different types of particle arrangement exist. They have also enabled a value to be determined for the isoelectric point of the edge surface of kaolinite.

The electrical double layers at edges and faces must differ either in the magnitude or in the sign of the surface potential in order to account for edge-edge and edge-face structures, since a face-face coagulated structure would be the condition of lowest free energy if the electrical double layers on all surfaces were identical and there was no potential energy barrier to coagulation. It is usually considered^{1,4} that the basal face of a kaolinite crystal is a surface of constant charge density, determined by the amount of isomorphous replacement within the crystal lattice; on the other hand, the edge surface has a surface potential which depends, as in the case of an oxidic surface, on the adsorption of the potential-determining hydrogen and hydroxyl ions². Schofield and Samson³ have demonstrated that at low pH values (below the isoelectric point of the edge surface) edge-face structures exist because the positively charged edges are electrostatically attracted to the negatively charged basal faces. At pH values slightly above the isoelectric point of the edge surface, however, the system becomes deflocculated at low ionic strengths, since the edge surface potential is negative and large, and results in high potential energy barriers to all types of coagulation.

The flow curves of clay suspensions frequently approximate to the Bingham model in which a certain yield value must be exceeded before flow is induced. The Bingham yield value is a function of the number and strength of the particle-particle linkages and is therefore a sensitive measure of the degree of coagulation. The addition of indifferent electrolyte reduces the Bingham yield value of edge-face coagulated systems by compressing the two electrical double layers and thereby reducing the electrostatic attraction. Conversely, the addition of electrolyte at high pH values increases the Bingham yield value by compressing all electrical double layers, thus lowering the energy barrier to van der Waals' coagulation. It can be argued that, at the isoelectric point of the edge surface, the system should already be fully coagulated in an edge-edge structure, even at low ionic strengths. Thus, the addition of indifferent electrolyte should have no effect on the yield value until sufficient electrolyte is added to promote face-face aggregation. If this argument is correct, then,

providing the ionic strength is not too high, plots of the Bingham yield value of kaolinite as a function of pH, each at a different ionic strength, should all coincide at one single pH value, the isoelectric point of the edge surface of a kaolinite crystal. This prediction has been validated by our study.

The rheological properties of suspensions containing 9 wt% of a highly crystalline kaolinite sample from a Cornish deposit, previously purified and converted to the homoionic sodium form according to the method of Schofield and Samson³, were investigated as a function of pH at different concentrations of NaCl (ref. 3). Flow curves were determined using a concentric-cylinder viscometer and in each case the data, determined at relatively high rates of shear, were extrapolated to give the Bingham yield value. Figure 1 shows the results, which are as predicted. The point of coincidence is at a pH value of 7.3 and we suggest that this is the isoelectric point of the edge surface of a kaolinite sample prepared by this method. It is clear that at pH values below this isoelectric point the yield value increases due to the onset of edge-face coagulation, whereas above it the system becomes progressively deflocculated. At pH values both above and below the isoelectric point of the edge surface, the effect of indifferent electrolyte is to change the yield value towards the value shown at the isoelectric point, that is, to promote edge-edge coagulation. This has been further corroborated³ by the fact that progressively higher electrolyte concentrations are required to achieve this yield value as the potential of the edge surface increases both above and below the isoelectric point.

The value of the isoelectric point obtained is comparable with the values obtained by others^{5,6}, and with the pH value of zero adsorption of hydrogen ions found by Flegmann *et al.*⁷. Flegmann *et al.*, however, suggested the isoelectric point of the edge surface of kaolinite to be at a pH value of 6, the maximum in the yield value-pH plot. This maximum was attributed to an edge-edge structure, but it can be seen from this work that this is a misinterpretation of the rheological data. Quirk⁸, however, has detected positive adsorption of chloride ions at pH values as high as 9-11 but it is not certain that a small amount of iron oxide impurity in his clay did not affect these results to some extent. Other values reported in the literature do not necessarily refer to clays prepared in the same way as reported here. As part of this study, certain commercial and natural clays have been investigated and the results compared with the model system described here. Different apparent isoelectric points were obtained which depended on the previous history of the clay samples. These results will be published elsewhere, along with a more detailed account of the influence of the electrical double layers on the rheological and sedimentation behaviour of the kaolinite sample reported here.

These results suggest that edge-face structures exist only below the isoelectric point of the kaolinite edge surface at low ionic strengths. Edge-edge structures exist to varying extents at all pH values at moderate concentrations (≈ 0.1 M in a 1:1 electrolyte), and at pH values around and slightly above the isoelectric point of the edge surface at low electrolyte concentrations. At higher electrolyte concentrations (> 0.1 M) it is suggested³ that face-face aggregates are formed at all pH values.

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Mechanism for shear delamination under dynamic loading

WE describe here a debonding mechanism for interfaces between dissimilar materials in conditions of dynamic loading, and we deal, in particular, with coated substrates that are impacted by solid or liquid projectiles. Protective coatings are commonly used to enhance the resistance of materials to impact erosion; for example, non-metallic fibre-reinforced composite materials are found to be very susceptible to impact erosion unless a suitable thin coating is applied. For these composites, certain elastomers can be successful as coating materials, even though they do not, intrinsically, possess high strength. Coatings of this type reduce the pressures that are transmitted to the substrate by presenting a lower dynamic impedance to the impacting body. The interfacial adhesion between the coating and substrate is of great importance because it enables the substrate to reinforce the coating by restricting the lateral strain, and therefore the stress, within the coating. When adhesion is lost, the coating itself becomes quickly eroded. Thus, the bond between the coating and substrate is often of great significance to the success of a coating–substrate system.

The impact of liquid drops on to a coated substrate has been considered by a number of authors^{1–3}, but little attention has been paid to the precise mechanisms of the adhesion breakdown between the coating and substrate. High speed photographic observations of the process of delamination under liquid impact^{4,5} and examination of

Fig. 1 Experimental arrangement for the high speed photography.

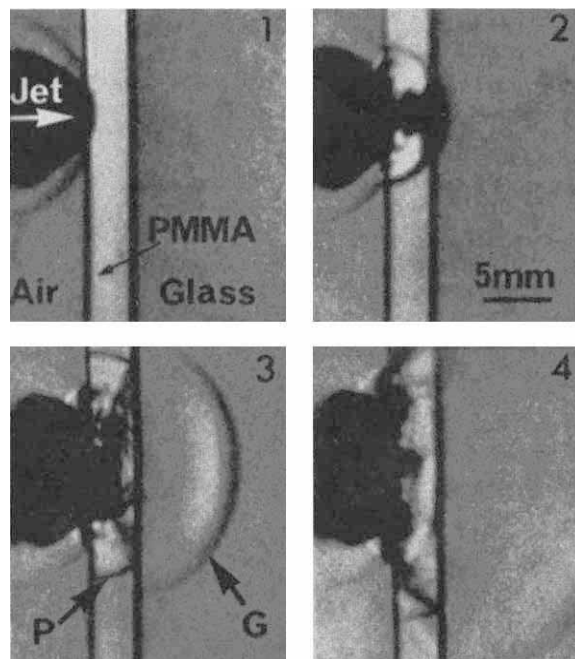
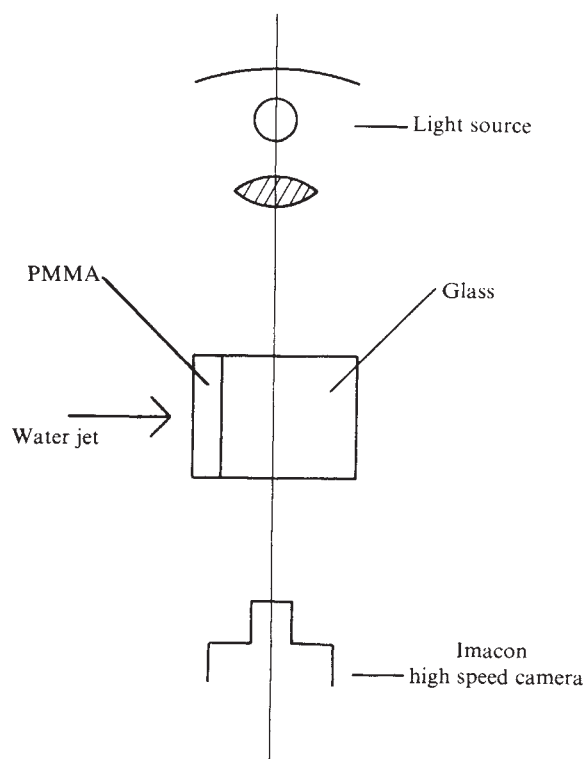


Fig. 2 The front of a water jet (750 m s^{-1}) impacting a PMMA layer that is bonded to a glass block. The jet head diameter is $\approx 3 \text{ mm}$. Frames are selected from an Imacon sequence of $10^6 \text{ frames s}^{-1}$. P, Compressive stress front in the PMMA; G, transmitted stress in the glass block.

impacted laminates have shown that the delaminations are usually composed of distinct coaxial annular regions. This suggests that several mechanisms are operative. Often the interface on the impact axis has not failed, and the total delamination is an annulus. This behaviour cannot be explained by the commonly cited mechanism of tensile failure resulting from reflected or dispersed stress waves, because such interactions lead to maximum tensions developing on the impact axis. Therefore the creation of shear stress along the interface must be considered. When stress waves from an impact meet an interface, shear stresses can be set up by several mechanisms; for example, they can arise from the incident pulse of shear stress (which is usually of a low magnitude) or from the oblique incidence of the main compressive pulse⁶. The high speed photographic sequences presented here illustrate another form of shear debonding of comparable magnitude to the latter. The mechanism arises from the wavefront separation that occurs when stress propagates parallel to an interface between two media which have different stress-wave velocities.

A schematic diagram of the experiment is shown in Fig. 1. The front of a water cylinder impacts a polymethylmethacrylate (PMMA) layer 3 mm thick, that is bonded to a glass block. The jet velocity in the experiments was 750 m s^{-1} (see also refs 7 and 8). A sensitive shadowgraph optical system⁴ was used to make the stress waves generated by the impact visible. The high speed photographs were taken with a Hadland Imacon framing image converter camera operating at $10^6 \text{ frames s}^{-1}$, and selected frames from two sequences are shown in Figs 2 and 3.

When the front of the jet first strikes the PMMA surface, the liquid behaves compressibly for about $1 \mu\text{s}$ and a very intense pressure, $\sim 1 \text{ GPa}$ is developed⁷. When the pressure front in the PMMA, P, reaches the PMMA–glass interface, it generates the transmitted pulse G in the glass. The stress propagation velocity in the glass is high ($5,600 \text{ m s}^{-1}$) compared with that in the PMMA ($2,700 \text{ m s}^{-1}$ to $\sim 3,200 \text{ m s}^{-1}$, depending on the stress level). Therefore, there is a critical

angle of incidence of the diverging pulse P beyond which no stress is transmitted across the interface. A non-delaminated region corresponding to the size predicted by this angle is found during high speed photographic observation of coated substrates'. Outside this diameter in the region of the interface, front G in the glass overtakes pulse P travelling in the PMMA: these separated fronts are seen clearly in frame 3 of Fig. 2. In frame 2 of Fig. 3 disturbance H, a 'headwave' in the PMMA, is generated by the faster stress front, G, in the glass. It indicates that a high stress is being transmitted across the interface by the separation of the wave fronts, that is, that shear strain is developed between stressed glass and unstressed PMMA. This is a potential delamination mechanism.

The theory of stress waves in these conditions has been developed by many authors (see, for example, refs 9 and 10). The conditions for delamination and its effect on the subsequent wave propagation are not, however, discussed. High speed photographic studies of spherical stress waves^{11,12} have shown that both the normal and tangential stresses at the interface achieve large values at various stages in the propagation history. The analogous situation of plane wavefronts propagating parallel to the boundary between coupled bars of dissimilar media has also been considered by a number of authors (see, for example, refs 13 and 14). They have shown that the coupling force can be of sufficient magnitude to cause debonding.

Observations of the formation of delamination fractures have shown that it is a very complex process, and involves many different mechanisms. Velocities of delamination growth exceed the maximum tensile crack velocities, and vary according to the stress wave propagation conditions. The simple mechanism illustrated here may not be the only one operating, but it does involve stress levels that are significant for many bonded interfaces. Even if it does not cause complete failure of the bond, it may cause nucleation of local failure sites which are then extended by the succeeding stress history.

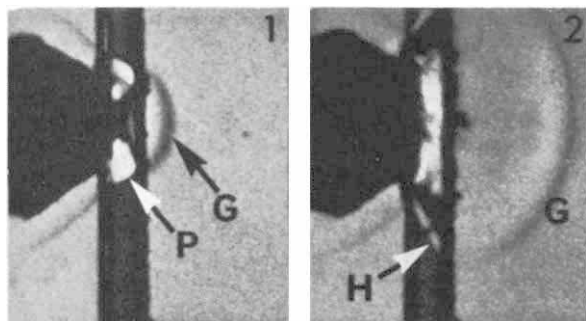


Fig. 3 As Fig. 2, but different illumination conditions also show the headwave, H. 1 μ s between frames.

An important point to note is that this mechanism will operate between media which are 'acoustically matched' (that is, media which have the same value for the product ρC , where ρ is the density and C is the stress wave velocity). Acoustic matching has often been considered as a means to minimise interfacial stress by eliminating reflected wave components. The shear mechanism of wavefront separation, which relies on a mismatch of C and not ρC , will, however, still in general operate. We have observed annular delaminations between such acoustically matched layers, which can be explained by this process. We conclude that, although acoustic matching of coatings may be beneficial, it is also important to choose materials which have similar values of C . In practice, this may be difficult to achieve by using a single coating material, but multiple coatings, with

a gradation of physical properties, could overcome this problem.

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Large scale changes in North Sea phytoplankton

CONTINUOUS plankton recorders (CPRs) are towed^{1,2} at approximately monthly intervals in the North Sea along standardised routes at a standard depth of 10 m. Analysis of long term results has revealed large scale trends and changes³. Here I deal with changes in the phytoplankton from 1948 to 1973 as evidenced by results from area C2 (Fig. 1) in the western North Sea.

As the CPR is towed through the water it filters the plankton on to a continuously moving band of silk with apertures of approximately 270 μ m. Phytoplankton is analysed in these samples in two ways. First, 'phytoplankton colour' is visually assessed into three categories⁴ to give a relative estimate of

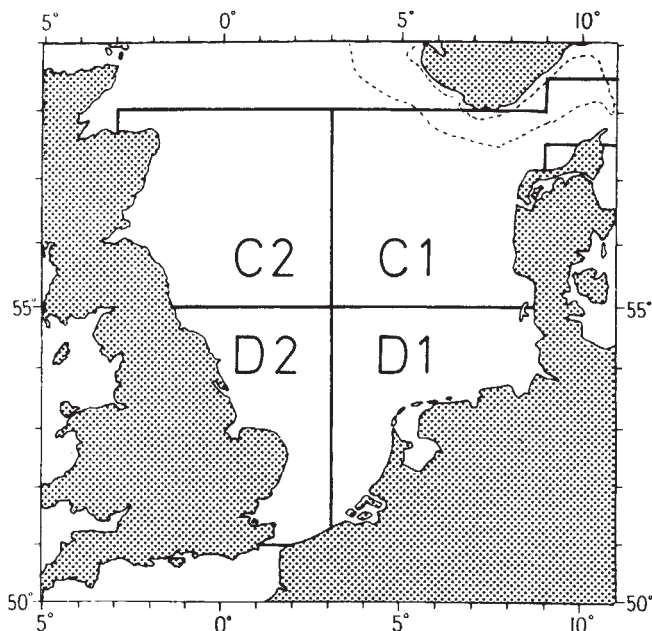


Fig. 1 North Sea standard areas.

Table 1 Species included in diatom and *Ceratium* spp. totals

Diatoms	<i>Ceratium</i>
* <i>Paralia sulcata</i> (Ehrenb.) Cleve	<i>Ceratium fusus</i> (Ehrenb.) Dujardin
* <i>Skeletonema costatum</i> (Grev.) Cleve	<i>Ceratium furca</i> (Ehrenb.) Clap. & Lachm.
* <i>Thalassiosira</i> spp.	<i>Ceratium lineatum</i> (Ehrenb.) Cleve
* <i>Dactylosolen mediterraneus</i> Perag.	<i>Ceratium tripos</i> (Müller) Nitzsch
* <i>Rhizosolenia imbricata</i> forma <i>shrubsolei</i> (Cleve) Schröder	<i>Ceratium macroceros</i> (Ehrenb.) Vanhöffen
* <i>Rhizosolenia styliformis</i> Brightw.	<i>Ceratium horridum</i> (Cleve) Gran
* <i>Rhizosolenia hebetata</i> forma <i>semispina</i> (Hensen) Gran	<i>Ceratium longipes</i> (Bailey) Gran
* <i>Rhizosolenia alata</i> forma <i>indica</i> (Perag.) Gran	
* <i>Rhizosolenia alata</i> forma <i>alata</i> Brightw.	
* <i>Hyalochaeta</i> spp.	
* <i>Phaeoceros</i> spp.	
* <i>Asterionella glacialis</i> Castr.	
† <i>Thalassiothrix longissima</i> Cleve & Gran	
* <i>Thalassionema nitzschioides</i> Hust.	
* <i>Nitzschia seriata</i> Cleve	
* <i>Nitzschia delicatissima</i> Cleve	

*All species typically form chains which increases their chance of being entrapped in the CPR silk.

†The large species *T. longissima* occurs free but frequently forms dense matted colonies.

All *Ceratium* species are large and likely to be well sampled by the CPR.

phytoplankton standing crop (visual chlorophyll). Second, since 1958, species have been identified and counted⁵. Most phytoplankton passes through the coarse meshes of the CPR but comparisons with water samples⁶ and the results of the survey indicate that the portion retained seems to reflect changes in the abundance and distribution of the major components of the phytoplankton. The large number of CPR samples taken over extensive areas smoothes the effect of patchiness and gives gross

estimates of abundance which are comparable within the CPR survey.

The monthly means for both methods of analysis are calculated for samples in 2° longitude × 1° latitude rectangles and then averaged into larger 'standard areas'. Results for Standard Area C2 are presented as contoured month/year diagrams in Figs 2–4. The pattern of phytoplankton change observed in Standard Area C2 is, in many respects, typical of the whole of the North Sea. *Ceratium* spp. and diatoms represent the totals of the average abundance of the seven most common *Ceratium* species and 16 diatoms in the North Sea (Table 1).

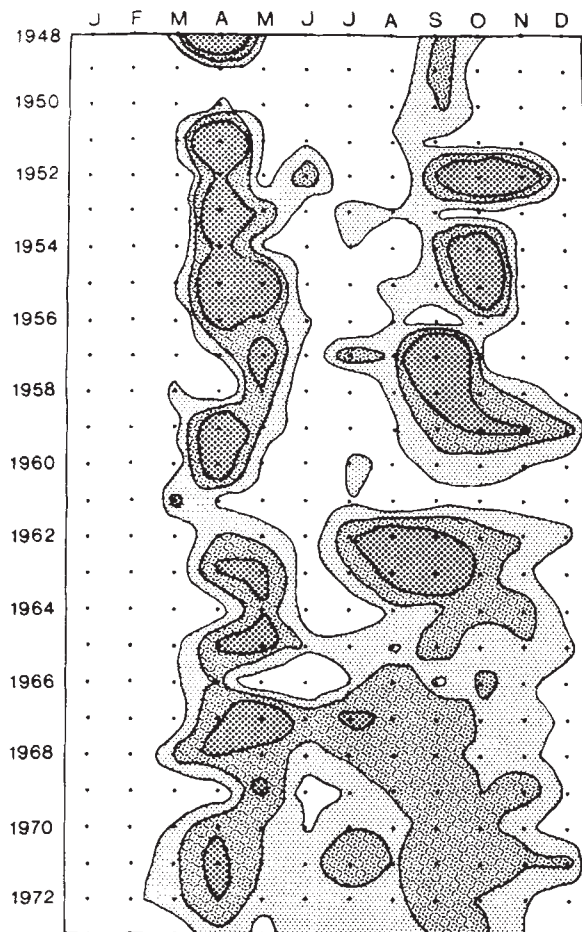
Before 1965, the analyses of phytoplankton colour revealed distinct spring and autumn blooms (Fig. 2) but after that date the distinction between the two blooms became less marked and the productive season became longer, although the autumn bloom declined in intensity. *Ceratium* spp. (Fig. 3) have shown little change in either timing or abundance since 1958.

The diatom count decreased markedly after 1965 (Fig. 4). The autumn bloom declined drastically and winter and summer diatoms almost disappeared. A more gradual but still considerable decline occurred in the spring bloom.

To some extent, there seems to be an inverse relationship between the pattern of phytoplankton colour and diatom changes. The diatoms have declined in abundance (especially in the summer, autumn and winter months) as phytoplankton colour has increased. Therefore, it seems that phytoplankton other than diatoms and *Ceratium* spp. as measured by the CPR has caused the general increase in colour. Other groups such as the silicoflagellates and coccolithophores only occurred in small numbers and *Phaeocystis* showed a decline similar to that of the diatoms after 1965. One possible explanation is that there may have been an increase in the abundance of micro-flagellates which partially disintegrate in formalin leaving their chloroplasts to add to the coloration of the silks. Such micro-flagellates would not be identifiable in CPR samples but could have caused the increase in phytoplankton colour. A similar replacement of a diatom by a flagellate population was described by Grøntved⁷ as part of the seasonal succession of the phytoplankton of the North Sea during 1947.

The pronounced change in the phytoplankton of area C2 which took place around 1965 is coincident with the return of plankton, which was typical of the 1920s, to the English Channel off Plymouth⁸. This reversal in Channel plankton was attributed by Russell *et al.*⁸ to climatic change which may have led to an increased Atlantic inflow into the North Sea. Unfortunately, the CPR survey did not commence in the North Sea until 1931 so that it is not possible to determine whether the recent

Fig. 2 Phytoplankton colour (visual chlorophyll) in Standard Area C2.



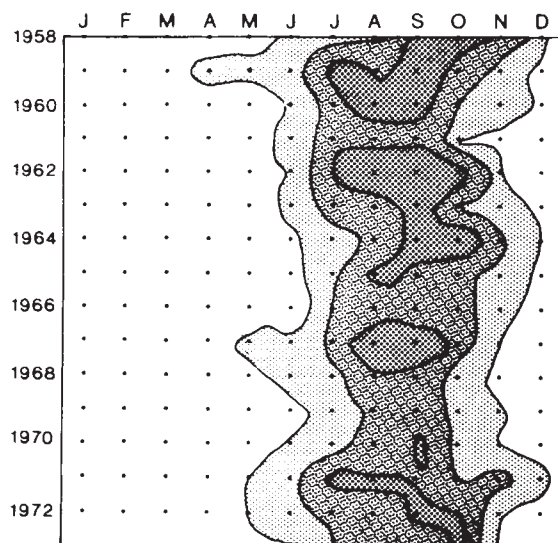


Fig. 3 Abundance of *Ceratium* in Standard Area C2.

changes in the North Sea are a return to the conditions of the 1920s.

If climatic changes are causing the considerable changes in the plankton of the North Sea and the English Channel, then they seem to be influencing the zooplankton and phytoplankton of the two areas in different ways; both zooplankton biomass and diatoms are increasing in the English Channel off Plymouth⁸, whereas in the North Sea they are declining³. But productivity as measured by ¹⁴C has increased in the English Channel since 1965 (ref. 8). While there are no comparable time series of productivity measurements in the North Sea, the standing crop as determined from crude estimates of phytoplankton colour certainly seems to have increased.

A second possible alternative cause of the phytoplankton changes is eutrophication caused by increasing nutrient levels⁹ in the North Sea. This seems unlikely, however, because eutrophication arising from pollution, only seems to be a potential problem in coastal waters¹⁰ and not in the offshore waters of area C2.

The changing composition of the phytoplankton in area C2 since 1965 is likely to be reflected at higher trophic levels. During the same period there has been a parallel decline in the

zooplankton biomass of the North Sea³. It is possible, therefore, that the plants which seem to be replacing the diatoms since 1965 may be less readily utilised as food. Contemporary changes have also taken place in the fish larvae of the CPR survey (S. H. Coombs, personal communication). We are now attempting to identify the organisms which seem to be replacing the diatoms and to determine whether the changes in the North Sea are cyclical and related to climatic change or to other as yet unknown influences.

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Is the illusory triangle physical or imaginary?

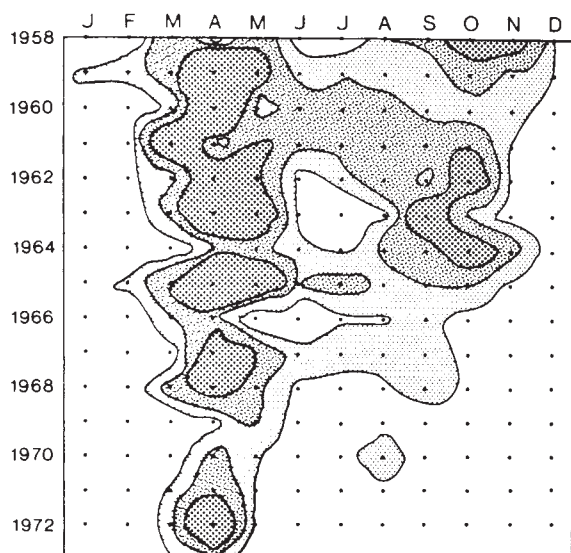
MOST pictures of visual interest contain a large amount of detailed information. Studies have shown that a great deal of these data can be filtered out without destroying a subject's or a machine's ability to recognise even quite complex objects^{1–4}. The resulting benefits are economy of memory store and considerable flexibility in the shape and detail of objects classified as the same.

Such filtering must to some extent distort our own visual perception and can account for certain geometric and contrast illusions⁴. There has been recent interest in illusory contours⁵. Figure 1a shows an example of an illusory contour perceived in a Kanizsa triangle. A black triangle is perceived as a unitary form separated in depth over a white triangle. The question now arises, what would happen if we filter this figure to get rid of the redundant information? In other words, what form remains if the detailed information (the higher spatial frequencies) not necessary for the identification of the original pattern features are removed?

This filtering was carried out as follows. The two-dimensional discrete Fourier transform of the original picture was made (Fig. 1b). (A log transform was used on both the transform and filtered pictures to compress the intensity levels and provide the maximum detail.) The magnitudes of these spatial frequencies were decreased according to the attenuation characteristics of the visual system⁶ (Fig. 1c). An inverse Fourier transform reconstructs this attenuated spatial frequency data (Fig. 1d). The filtered picture results in smoothed pattern features and a loss in contrast in the lines, but enough resolution is still retained to provide an illusory triangle. There is still no clue from this picture, however, as to how the original pattern features interact to provide the illusory triangle.

Further spatial filtering was carried out to remove even more redundant pattern information. Previous experiments on filtering suggest that the low spatial frequencies will provide sufficient information for classifying forms^{1–4}. When this filtering was accomplished by removing three-fourths of the higher spatial frequencies (Fig. 1e), we note a generally homogeneous dark triangle surrounded by a somewhat homogeneous lighter

Fig. 4 Abundance of diatoms in Standard Area C2.



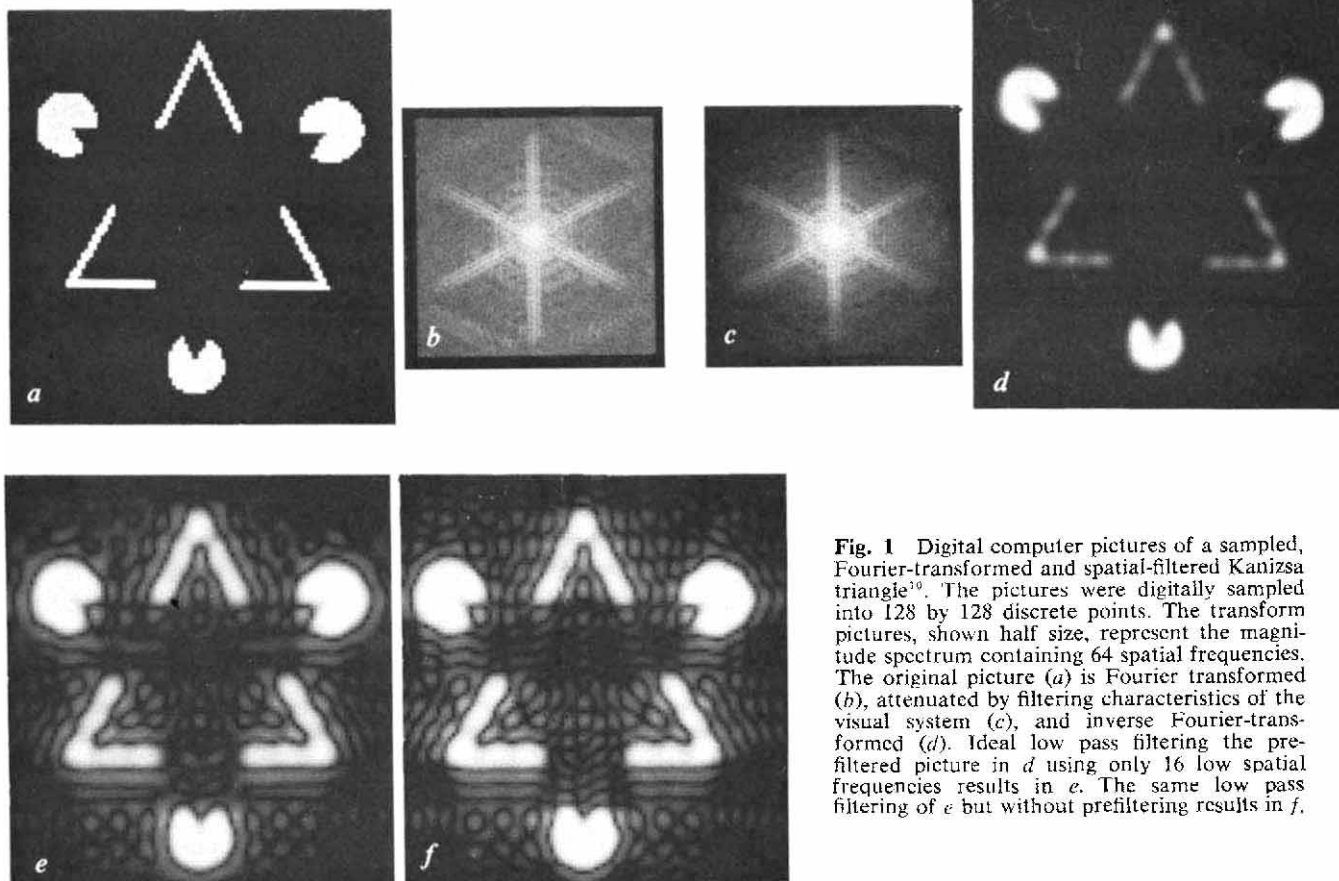


Fig. 1 Digital computer pictures of a sampled, Fourier-transformed and spatial-filtered Kanizsa triangle¹⁰. The pictures were digitally sampled into 128 by 128 discrete points. The transform pictures, shown half size, represent the magnitude spectrum containing 64 spatial frequencies. The original picture (a) is Fourier transformed (b), attenuated by filtering characteristics of the visual system (c), and inverse Fourier-transformed (d). Ideal low pass filtering the pre-filtered picture in d using only 16 low spatial frequencies results in e. The same low pass filtering of e but without prefiltering results in f.

triangle and area. Note in particular the top dark horizontal boundary line of the dark triangle. (This sharp boundary results from the most linear sectorised disk and smooth line features of the original pattern. Other similar pattern features of the original triangle are jagged because of the coarse quantification process, and result in less well defined regions in the filtered pictures. Further improvements in techniques will provide even more striking results.) It seems that as in previous results, limited regions of low spatial frequencies do provide pattern information that is not readily apparent from analysing the original pattern features.

Have the initial filtering characteristics of the visual system attenuated the low spatial frequency information in such a manner as to aid the detection of the 'illusory' triangle? We answer this question by low pass filtering the original picture directly. The resulting picture (Fig. 1f) shows a somewhat less homogeneous dark triangle region. This result suggests that the low spatial frequency attenuation characteristics of the visual system do aid the formation of the 'illusory' triangle.

To convince a blind mathematician that a triangle really exists, one could have simply correlated the original pattern with an actual triangle. Correlation would provide an independent and objective measure of the degree to which the illusory triangle exists in the original pattern. Previous pattern classification studies, however, have shown that the low spatial frequencies provide generalised form information that is relatively insensitive to discriminable differences between objects classified as the same. Therefore, to be consistent with previous analysis and not treat this pattern in any way different from others, we compared just the low spatial frequencies of the original pattern with the low spatial frequencies of a black triangle of similar size and orientation. They correlate.

It should be stressed, however, that not all subjective contour patterns may be explained so easily by such filtering or correlation techniques. If the sectorised disks are replaced with small dots, as Gregory has done, an illusory triangle does exist although it is markedly less evident⁸. Initial filtering analysis of a similar pattern has yielded promising but not too convincing

results, because of the relatively small energy contained in the dots. Again, further research will be needed to improve techniques and determine whether or not additional mechanisms are being used to detect and process other illusory contours.

In summary, the Kanizsa triangle has been filtered in the above well defined manner. The final picture contains a black triangle, but mathematically we have not informed the system used about a triangle. We have not explicitly manipulated the original pattern features, nor have we provided additional cognitive clues. Neither the Fourier transform nor the filtering acting on the original pattern have generated the illusory contour triangle. Rather, the filtering has isolated and enhanced the illusory triangle information that was implicit in the overall spatial relationships of the original pattern features. Therefore the original picture must have contained the triangle. It is therefore not surprising that our visual system detects it. This analysis is consistent with visual spatial information being filtered by quasi-independent spatial frequency-orientation channels as evidenced by neurophysiological and psychophysical studies⁷⁻⁹.

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Intrauterine nutrition and aggression

WITH the increase in conflict and aggression in human society, the underlying causes of aggression take on added significance. The laboratory investigation of human aggression is difficult primarily because of ethical considerations. Animal aggression must therefore serve as a model for the experimental study of suspected causes of aggression. One potential cause is malnutrition, which has been assessed by studies of the effects of prenatal zinc deficiency and intrauterine undernutrition on aggressive behaviour in the adult rat. Prenatal zinc deficiency was chosen because it has been found to significantly reduce the size of the brains of foetal¹ and neonatal rats², to impair the synthesis of DNA in the rat brain³, and to decrease the activity of RNA polymerase in neonatal rat liver⁴ and brain⁵. When zinc deficiency is very severe an increased incidence of central nervous system teratology occurs in the foetal rat⁶. Behavioural deficits such as impaired avoidance learning have also been observed^{1,7}.

tory chow⁸ *ad libitum* and tap water. After delivery, all the dams and their pups were continued on the chow *ad libitum* and tapwater. The reduced food consumption during the zinc-deficient period had a marked effect on the mean weights of the ZD and PF dams (Fig. 2). For the first 40 d of life, the ZD pups were smaller than the PF and AL pups, but by day 75, there were no differences among the three groups (Table 1).

When the pups were 75 d old, 10 females from each group were randomly selected and tested for aggression. The 10 females in each group were divided into five pairs for a total of 15 pairs. Each pair was tested for aggressive behaviour in a clear Plexiglas chamber (32.0×25.5×30.5 cm). The grid floor consisted of 0.3-cm stainless steel rods spaced 1.9 cm from centre to centre. The rods were electrified by a Grason-Stadler Model 700 shock generator. The chamber was housed in a box 80 cm high, 61 cm deep and 80 cm wide. Two fans provided intake and exhaust ventilation. Each pair of rats was given 25 shocks per d for 10 d. A modified counter balance procedure (ABBA) was used. On the first 2 d of shock, the intensity was 1.6 mA followed

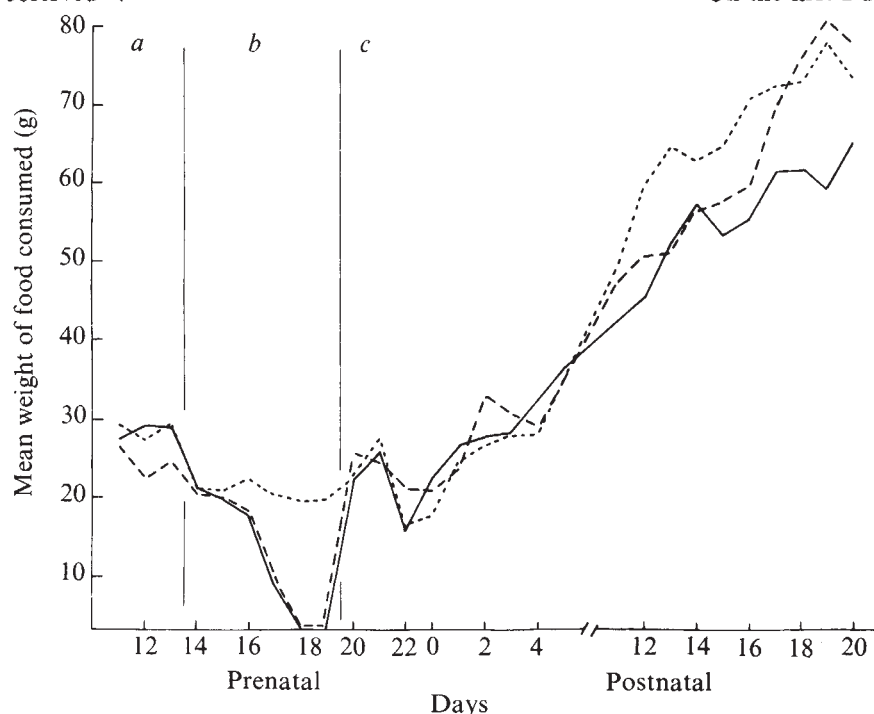


Fig. 1 Comparison of food consumption among three groups of dams. AL dams (·····) ate significantly more food ($P < 0.001$) than the ZD dams (—) during the zinc-deficient period. PF dams (---) were given the same quantity of food as eaten by the ZD dams during this period. There were no differences among the three groups during the normal diet periods. Day 0 is the day of delivery. a, Normal diet; b, zinc-deficient diet; c, normal diet.

This report shows that adult female rats whose dams suffered prenatal zinc deficiency (ZD) or undernutrition as a result of pair feeding (PF) are significantly more aggressive at a high level of shock (1.6 mA) than adult female rats whose dams were fed *ad libitum* (AL) throughout pregnancy; and that the ZD female rats are more aggressive than the PF female rats. At a lower level of shock (1.3 mA), PF and AL female rats display a similar level of aggression well below that of the ZD female rats.

Thirty pregnant Long-Evans rats were randomly divided into three groups. The dams were housed in plastic cages in a room that was controlled for temperature and humidity. Starting on day 14 of pregnancy, 10 dams (ZD) were fed a biotin-enriched sprayed egg-white diet which contained less than 1 µg zinc per g (ref. 2) and deionised water. As zinc deficiency causes anorexia, a second group of 10 dams was individually paired (PF) with the ZD dams and fed the same quantity of the diet as was consumed by their ZD partners (Fig. 1). The PF dams were supplemented with 50 µg zinc per ml of drinking water. A third group of 10 dams (AL) were fed the same diet *ad libitum* and given the zinc-supplemented water. On day 20 of pregnancy, all dams were taken off the zinc-deficient diet and fed Purina labora-

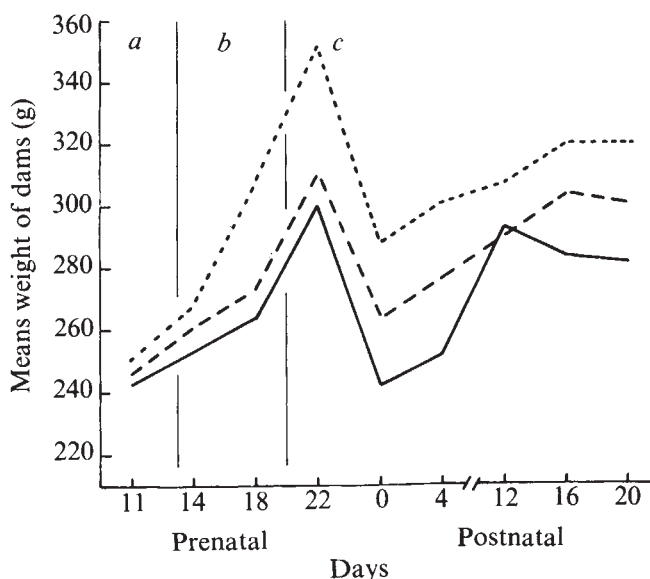


Fig. 2 Mean weight of dams during the prenatal and postnatal period as a function of dietary manipulations during days 14–20 of pregnancy. See Fig. 1 for coding.

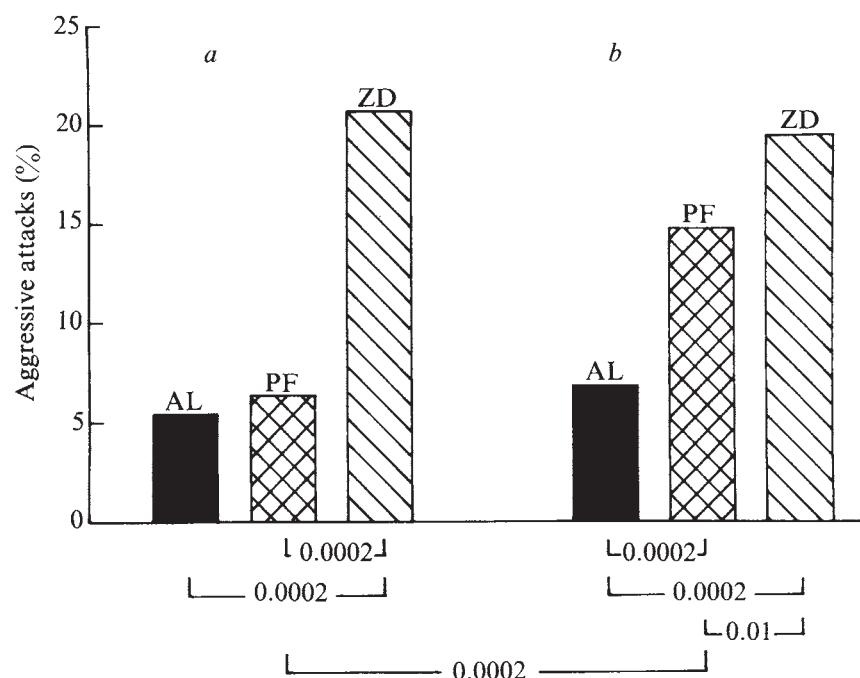


Fig. 3 Comparison of the level of aggression among the three groups of rats at two different levels of shock. ZD rats were significantly more aggressive than either PF or AL rats at both levels of shock. PF rats were significantly more aggressive than the AL rats at 1.6 (b) but not at 1.3 mA of shock (a).

by 5 d of shock at 1.3 mA. The shock was 1.6 mA for the final 3 d. All rats were given 1 d rest after each day of shock. All shocks were 0.5 s in duration and spaced 5.0 s apart. The sessions were videotaped and later evaluated independently by three judges who counted aggressive attacks per pair per session. Aggressive attacks were defined as 'directed movement toward the opponent' which resulted in contact, including at least one of the following responses: biting, sparring, upright attack posturing, or supine submissive posturing adopted by the attacked rat⁸. All judgments were made independently and without knowledge of the group origin of each pair. The reliability between the judges, computed by analysis of variance estimate of reliability⁹, for each day, ranged from 0.95 to 0.99 for the 10 d.

Figure 3 shows the percentage of aggressive attacks displayed by the three groups for the two levels of shock. The total percentage for each group represents the percentage of shocks resulting in aggressive attacks observed in each of the five pairs over 125 trials or 625 trials for the group. Differences between these proportions were compared for statistical significance using a grand combined proportion of aggressive attacks in the error term¹⁰. The level of significance between groups is shown in brackets below the group columns. Only those probabilities $P \leq 0.10$ are shown.

The results show that the AL rats were significantly less aggressive than the ZD rats regardless of the level of shock. Furthermore the levels of aggression remained unchanged for both groups even though the level of shock changed. The offspring of the PF rats who had suffered intrauterine undernutrition¹, were highly aggressive at a high level of shock but were no more aggressive than the AL rats at a lower level of shock. These results suggest that animals that experience prenatal undernutrition during the critical period for brain growth will be more aggressive than normal animals. The results also suggest that animals that experience zinc deficiency during the same period of intrauterine

development are more severely impaired and are even more aggressive than prenatally undernourished rats. Although extrapolation to human aggression is premature, these results do suggest the need for additional research in the field of malnutrition and aggression.

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Induction of plants from pollen grains of *Petunia* cultured in vitro

THE ease with which plantlets can be produced from somatic tissue, protoplasts and anther cultures of *Petunia*¹⁻³ has prompted us to use isolated pollen grain culture⁴ to determine precisely the stage at which pollen elicits the best androgenic response, with a view to increasing the yield of haploid plants thus produced.

Flower buds in various stages of development from two strains of *Petunia hybrida* L. (Cascade and Rose du Ciel) grown in the greenhouse (the phytotron at Gif-sur-Yvette) were sterilised in a filtered suspension of calcium hypochlorite (7%)

Table 1 Mean weights (g) of pups at 0, 20, 40 and 75 d of age

	0	20	40	75
ZD	5.48	29.8	107.8	224.6
PF	6.09	36.3	129.6	220.6
AL	6.11	37.5	131.2	219.3

for 5 min and rinsed three times with sterile distilled water. Fifteen to twenty anthers were homogenised in a Potter-Elvehjem homogeniser tube with 10 ml of culture medium. The suspension thus obtained was filtered through two superposed sieves of 53 and 73 mesh. New medium was added to give the final concentration desired. This inoculum contained microspores and/or pollen grains according to the age of the anthers used. A large amount of pollen could thus be obtained. The inoculum was rinsed three times with new medium after centrifugation at 120g for 5 min and resuspended in the fresh medium. The pollen grains were cultivated in plastic Petri dishes (6 cm diameter) containing 3 ml of solution inoculated with 10^4 pollen grains per ml. The dishes were maintained at 25 °C for 7 d in the dark and then illuminated continuously with 1,000 lx at a temperature of 28 ± 1 °C. The culture medium consisted of macroelements according to Murashige and Skoog (we used half the concentration described in ref. 5), the microelements and vitamins of Nitsch and Nitsch⁶ supplemented with auxin, cytokinin, boric acid (25 mg l⁻¹), sucrose (2%) and glucose (2%). The pH was adjusted in all cases to 5.6 before autoclaving.

Greenish calluses appeared gradually from the pollen 10–20 d after inoculation into the medium containing various combinations of benzyladenine (BA) and naphthalene acetic acid (NAA). As Table 1 shows, that calluses formed in the combination of BA (0.1 mg l⁻¹) and NAA (0.1 mg l⁻¹) were much more numerous than in other combinations tested. After 30 d of culture 50 pieces of pollen-callus from *P. hybrida* Rose du Ciel were transferred to the same medium; 30 of them gave rise to shoots, 12 produced compact green calluses and 8 did not differentiate.

Fig. 1 *a*, A pollen plant of *P. hybrida* L. Rose du Ciel ($n = 7$), ($\times 1$). *b*, pollen stages: *b*, binucleate; *m*, at first haploid mitosis; *u*, uninucleate ($\times 800$). *c*, and *d*, dividing pollen grains (arrows, *c*, 1 week of culture, $\times 300$; *d*, 2 weeks of culture, $\times 220$); *e*, pollen calluses after 30 d ($\times 1.5$); *f*, shoots from pollen callus after 45 d ($\times 1.5$).

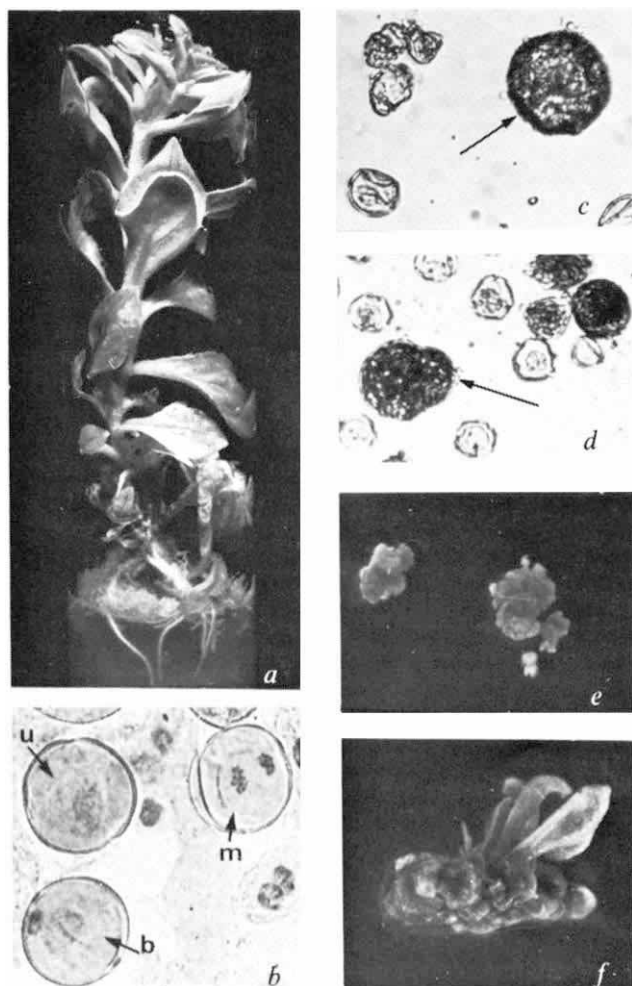


Table 1 Effect of different concentrations of NAA and benzyladenine on formation of pollen calluses in *P. hybrida* L.

Composition of the medium:		No. of calluses formed from isolated pollen grains	
NAA (mg l ⁻¹)	Benzyladenine (mg l ⁻¹)	Rose du Ciel	Cascade
0.1	0.1	2	0
0.5	0.5	13	8
0.1	1	61	58
1	0.1	32	36
1	1	25	30

Pollen extracted from 300 anthers at first pollen mitosis was inoculated for each treatment. Results were scored after 1 month of incubation.

Roots were initiated when the shoots were transferred into basal medium with indolyl-3-acetic acid (0.1 mg l⁻¹) or NAA or without growth hormones. Once they have formed an adequate root system the plantlets (Fig. 1) can be transplanted to pots and raised to mature plants. The same result was obtained when *P. hybrida* Cascade was tested for organogenesis.

The pollen stage at the time of inoculation is an important factor, determining the success or failure of pollen culture. According to our results the microspore inoculated at an early, mid or late uninucleate stage did not divide in any medium or combination tested. Pollen inoculated at, just before or just after the first mitosis (Fig. 1*b*), however, responded to the various media tested with essentially the same results for the two varieties (Table 1).

The course of callus formation in androgenic pollen of *P. hybrida* Rose du Ciel cultured *in vitro* was also followed. Some pollen grains (5–10%) enlarged considerably during the first week of culture (Fig. 1*c*) to form round multicellular masses (Fig. 1*d*), whereas others (90–95%) degenerated progressively. During the third week of culture the round multicellular masses produced irregular calluses (Fig. 1*e*). The formation of many shoot apices could be observed on the same callus within 40 d but only some of them formed shoots after prolonged incubation in the culture medium or after transfer to fresh medium (Fig. 1*f*). As these data show, only a small percentage of pollen grains *P. hybrida* developed into plants—approximately one plant developed from the pollen of eight anthers (that is 20% androgenic anthers). This, however, was a greater percentage of plant morphogenesis than Raquin and Pilet achieved with anther culture (1% androgenic anthers)³.

Cytological examination of the root-tip squashes of regenerated plants revealed haploid, diploid ($2n = 14$) and triploid plants among the population. The frequency of haploid plantlet formation was generally low compared with diploid or triploid; of 30 plants initiated from pollen culture, three were haploid, seven diploid and 20 triploid in *P. hybrida* Rose du Ciel. The existence of ploidy differences among the adult population is common in plantlets derived from microspores and can be accounted for by endomitosis or nuclear fusion: in such cases they will be homozygous^{7–10}. Thus, homozygous diploids obtained by pollen culture could eliminate long delays in the production of inbred lines.

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Nerves and angiogenesis in amphibian limb regeneration

In the past 150 yr, since the requirement of nerves for amphibian limb regeneration was demonstrated, there has been considerable progress in specifying the nature of the phenomenon but virtually none in understanding the site or nature of the neuronal effect¹. If denervation is carried out early, amputated limbs will not regenerate and may even undergo regression; at later stages in regeneration, when a blastema has formed, regeneration is not prevented by denervation, although in the newt at least, growth in volume is inhibited while growth in length and morphogenesis continue^{2,3}. Studies on the neuronal dependence have shown that any nerve can provide the trophic influence, that neither central nor reflex circuitry is required, and that a threshold number of nerves at the amputation surface is needed⁴. The neurotrophic agents do not determine the nature of the regenerate but only its growth properties⁴, and this is borne out by many biochemical studies, all of which suggest that nerves nonspecifically affect macromolecular synthesis and cell division^{5,6}.

Folkman's work on tumour angiogenesis⁷ has emphasised the importance of vascularisation and blood supply in growth processes, and how little we know of the mechanisms involved in angiogenesis in normal development. He has shown that tumours will not grow larger than about 1–2 mm unless vascularised. This suggested to us a new interpretation for the effect of nerves on limb regeneration. Our hypothesis is that the trophic action of the nerves is not on the growth of the regenerate itself but that the nerves are necessary for the vascularisation of the blastema. Thus, early denervation will prevent regeneration by blocking vascularisation.

The vasculature of regenerating limbs of the newt (*Triturus*

Fig. 1 Vascular changes during limb regeneration in the newt *T. cristatus*. *a*, 7 d after amputation. *b*, pre-small cone phase (10 d after amputation): the avascular rim is now about 0.5 mm wide. The whole mounts are difficult to photograph satisfactorily, low lighting being needed to see the otherwise transparent tissues. When viewed end-on, the avascular tip is very clear. *c*, Small cone blastema: this is now beginning to show signs of vascular buds growing into the proximal parts of the blastema. The stump boundary is shown by the arrows. *d*, Flat-cone blastema: the blastema is now fully vascularised and some thicker vessels are beginning to appear. ($\times \sim 9$).

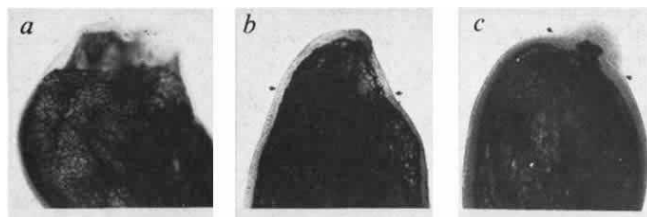
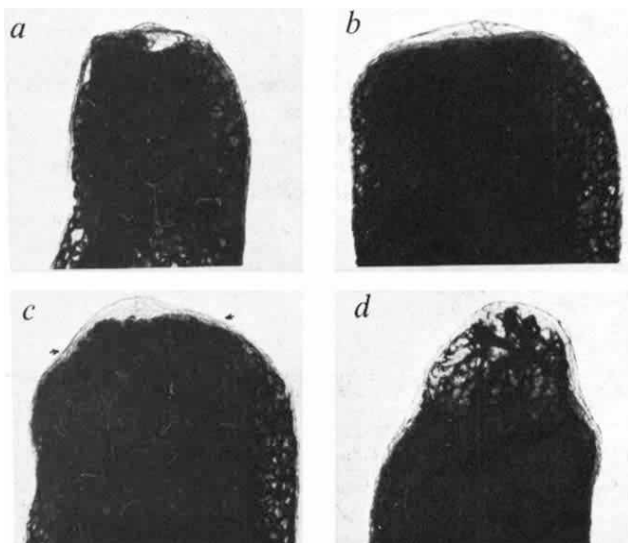


Fig. 2 Vascular pattern of regenerating and of denervated limbs of the axolotl *A. mexicanum*. *a*, 7 d after amputation: this shows clearly the retracted dermal vasculature, leaving a little more than 1 mm of avascular tissue more distally. The fine anastomosing capillary network can be seen easily. *b*, The innervated (control) limb after 17 d of regeneration. *c*, The limb denervated 3 d after amputation and left for 2 weeks to grow (17 d in total). The control side has formed a highly vascularised large blastema (more than 1.5 mm), whereas the denervated side has formed a small avascular blastema (0.5 mm). (The denervated side shows a small haematoma underneath the blastema. This was more common in denervated animals. Whether this is due to fragility of the vasculature or a loss of the ability of vasoconstriction of the ends of the vessels because of denervation is not known.) ($\times \sim 12$).

cristatus) and axolotl (*Ambystoma mexicanum*) was investigated by injecting Indian ink through a fine glass pipette into the hearts of MS222-anaesthetised animals. The limbs were then amputated, fixed in Bouin's, dehydrated and cleared in methyl salicylate. This technique is crude and cannot be expected to reveal subtle changes in the blood supply, but gross changes have been observed. The vasculature of the whole arm can be divided roughly into two main components. The first appears to be situated in the dermis in close proximity to the surface, forming a sheath over the whole arm. It is made up of a very fine anastomosing meshwork of vessels (10–30 μ m diameter), and gives the appearance, in an Indian-ink injected animal, of lace gloves (see Fig. 2*a*). These vessels can be shown histologically to surround the numerous dermal glands. The second component comprises all the deeper-lying large vessels that supply the other tissues of the arm. These tend to be coarser in appearance except for the fine vessels in the muscles. These are, however, easily distinguishable due to their parallel arrangement. After amputation, the skin contracts away from the cut surface, taking with it the underlying dermal vasculature (Fig. 2*a*). Thus, in the first few days of regeneration there is a region immediately below the wound epithelium that is avascular. The wound epithelium thickens to form an apical cap and blastema formation starts⁸. The pre-small cone phase is avascular, but by the small cone stage, small capillary buds are beginning to sprout and invade the proximal blastema (Fig. 1). These are fine vessels and can be traced to the dermal network of capillaries as well as the deeper vessels. The distal part of the small cone blastema still appears to be avascular and these tiny vessels do not get to the distal margin until the large cone stage. The avascular early blastema is more striking in the axolotl than in the newt (Fig. 2*a*). After the large cone stage, bigger vessels are quite often seen in the blastema. The distal margin of the large cone blastema has a continuous capillary supply underneath it. After the large cone stage, this vascular margin becomes fragmented as the delineation of the digits is made, the avascular spaces corresponding to the tips of the future digits. Tubes of vessels form as the indentations mark off externally the four digits. This description is essentially the same as that of Peadar and Singer⁹ for the newt *Triturus viridescens*.

Vascularisation was also investigated in limbs denervated at various stages and at successive intervals after denervation. Both forelimbs were amputated but only one side was denervated, through the brachial plexus, the other serving as a control. If denervation was carried out before the blastema became vascularised, the blastema did not become vascularised during the following 2 weeks whereas the control limbs regenerated normally (Fig. 2*b* and *c*). Denervation at the time when the

blastema begins to become vascularised, or later, did not appear to have gross effects on vascularisation but the resultant blood vessels tended to be thicker and the fine dermal network was often absent.

These results are consistent with our hypothesis that vascularisation of the blastema is dependent on the presence of nerves and that vascularisation plays a crucial role in normal regeneration. We can thus envisage the following interactions in regeneration. After amputation the retraction of the dermal vasculature results in an avascular region at the end of the stump. It is this region which undergoes dedifferentiation and gives rise to the blastema. We suggest that the blastema will not grow unless it becomes vascularised and that this vascularisation requires the presence of nerves. This accounts for the behaviour of limbs denervated at an early stage of regeneration. Denervation at stages when the blastema is already vascularised should have no effect on regeneration, unless the continued presence or further growth of the vessels is still nerve dependent. In fact the reduction in growth is associated with a reduction in vascularity. Here, muscle is more likely to be affected than cartilage, and histological investigations have shown this to be so¹⁰. Our hypothesis requires that limbs denervated very early should develop to the stage of a small avascular blastema and this is what both we and Revardel and Chapron¹¹ have found (Fig. 3). Our interpretation also explains why the effects of denervation are nonspecific and simply result in a general

angiogenesis and whether their effect is on the growth and sprouting of vessels themselves, or on tissues they invade. What is clear, is that blood supply and angiogenesis may play a crucial role in limb regeneration.

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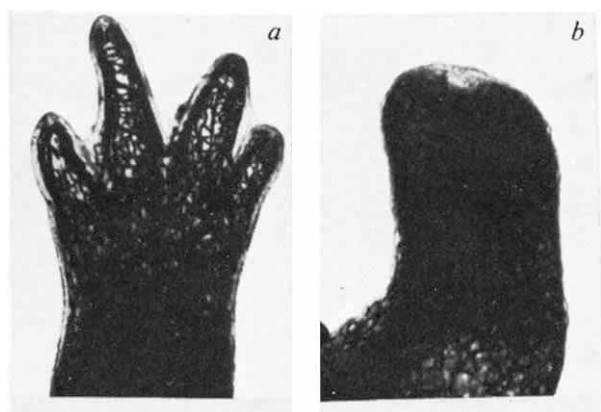


Fig. 3 Vascular pattern of regenerating normal and denervated limbs of the newt *T. cristatus*. *a*, The innervated control limb. *b*, The limb denervated during the pre-small cone phase (about day 10 after amputation) and left to grow for 2 weeks, that is, for the same period as the control. The control side has formed a highly vascularised four digit blastema, whereas the denervated side has remained in the avascular pre-small cone phase. ($\times \sim 15$.)

decrease in cell division and macromolecular synthesis at all stages. Moreover, the transitory increase in cell division and macromolecular synthesis immediately after denervation⁶ could be due to vasodilation caused by denervation. Blood supply may also be involved in the dedifferentiation of stump tissues. Dedifferentiation occurs in the avascular tip and we have shown that bone regression of about 0.5 mm occurs⁸ and this is similar in depth to the avascular region. Revardel and Chapron¹¹ have also emphasised the correlation between dedifferentiation and avascularity. It is also possible that the effects of X irradiation on regenerating limbs are in part a result of effects on the blood vessels.

The idea that blood supply is important in regenerative processes is not new and has been invoked in relation to compensatory hypertrophy of organs such as liver and lung¹²; the novel feature suggested here relates to the role of nerves. While all the above makes our suggestion plausible it must be emphasised that it in no way proves it: it is in fact disconcerting that we have not been able to find any reports relating nerves to angiogenesis in other systems. If we are correct, however, one may begin to consider how nerves could affect

Permeability of solutes through biological membranes measured by a desorption technique

THE influence of molecular structure of a solute on its permeation through biological membranes has been evaluated by various methods^{1–4}. For permeation of a solute across a membrane of thickness, h , the permeability coefficient, P , can be expressed in terms of the membrane-solution partition coefficient (K_m) and the diffusion coefficient in the membrane (D):

$$P = K_m D / h \quad (1)$$

We now present a further method, based on a desorption technique, in which the diffusivity of a solute in a membrane and its membrane-solution partition coefficient can be determined directly. A similar technique has been used in studies of vapour diffusion in polymers⁵. Biological material, initially equilibrated with an aqueous concentration of solute, is immersed in distilled water. The rate at which a solute is desorbed from the membrane reflects its diffusivity whereas the amount desorbed is used in the estimation of the membrane-solution partition coefficient of the solute.

The approach has several advantages. (1) Direct estimation of membrane-solution partition coefficients (which are not affected by the irreversible sorption of the solute on the membrane) avoids analysis of permeation results by organic solvent-water partition coefficients⁶; (2) the diffusivities of different solutes in a membrane are indicated by the rates of desorption of the solutes and may be compared without estimates of membrane thickness; (3) the results of this technique are relatively insensitive to the presence of holes or tears in the membrane; and (4) the presence of complications such as aqueous boundary layer effects⁷ or additional routes of solute penetration^{1,4} may be ascertained by comparison of the permeability estimates from permeation⁸ and desorption experiments.

The several treatments of diffusion (random walk, solution of formal diffusion equations, Eyring's theory and so on) are compatible and lead essentially to the same solution⁹. Crank¹⁰ presented two solutions for the diffusion of a solute into or out of a semi-infinite slab with boundary conditions of in-

stantaneous equilibrium of sorbent entities at the surfaces. The solutions are given as:

$$\frac{M_t}{M_\infty} = 1 - \frac{8}{\pi^2} \sum_{n=0}^{\infty} \frac{1}{(2n+1)^2} \exp \left[-\frac{(2n+1)^2 \pi^2 D t}{h^2} \right] \quad (2)$$

and

$$\frac{M_t}{M_\infty} = 4 \left(\frac{Dt}{h^2} \right)^{1/2} \left[\frac{1}{\pi^{1/2}} + 2 \sum_{n=1}^{\infty} (-1)^n \operatorname{ierfc} \left(\frac{nh}{2(Dt)^{1/2}} \right) \right] \quad (3)$$

where M_t is the amount of solute sorbed or desorbed at time t , M_∞ is the equilibrium amount sorbed or desorbed after infinite time, D is the diffusion coefficient of the solute in the membrane and h is the membrane thickness.

These equations are valid for the desorption of a solute from a biological membrane providing several assumptions are satisfied. (1) There is a single solute in true solution and initially uniformly distributed in the membrane; (2) the composition of the membrane is unaltered during the diffusion process; (3) the diffusion coefficient of the solute is independent of time and position in the membrane, and (4) the solute is desorbed into a well stirred infinite sink which does not affect the desorption process.

If the solute is desorbed from a membrane into a well stirred sink, a contribution to M_t and M_∞ is given by M_i the solute present in solution adhering to the surfaces of the membrane. This may be reduced by rinsing the surfaces of the membrane. In this study the spectrophotometric analytical procedure has introduced a lag time (τ).

Addition of the parameters τ and M_i to the equations yield:

$$M_t = M_\infty - \frac{8(M_\infty - M_i)}{\pi^2} \times \sum_{n=0}^{\infty} \frac{1}{(2n+1)^2} \exp \left[\frac{(2n+1)^2 \pi^2 D(t-\tau)}{h^2} \right] \quad (4)$$

and

$$M_t = M_i + 4(M_\infty - M_i) \left(\frac{D(t-\tau)}{h^2} \right)^{1/2} \times \left[\frac{1}{\pi^{1/2}} + 2 \sum_{n=1}^{\infty} (-1)^n \operatorname{ierfc} \left(\frac{nh}{2D^{1/2}(t-\tau)^{1/2}} \right) \right] \quad (5)$$

The amount desorbed (M_t) is nonlinear with time (t) with four unknown parameters τ and M_∞ , M_i and D/h^2 . Since approximate values of τ and M_∞ are known, estimates for M_i and D/h^2 may be found using an approximation of equation (5) which applies over short times:

$$M_t = M_i + 4(M_\infty - M_i) \left(\frac{D(t-\tau)}{\pi h^2} \right)^{1/2} \quad (6)$$

With these initial estimates, an iterative nonlinear least squares regression program (NONLIN, C. M. Metzler, Upjohn, which uses a modification of Hartley's¹ method to estimate the parameters of a system of nonlinear functions) was used. The best parameter values were found by minimising equation (7) until convergence (where lack of change in sums of squares (SS) is the convergence criterion):

$$SS = \sum_{i=1}^n \left(M_{t_i \text{ observed}} - M_{t_i \text{ predicted}} \right)^2 \quad (7)$$

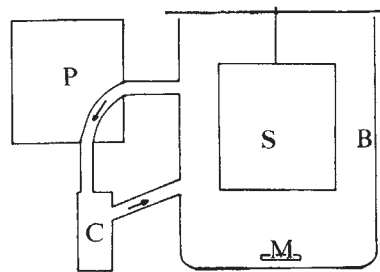


Fig. 1 Arrangements for monitoring the desorption of a compound from a biological membrane. Compound is desorbed from the membrane held in mesh support, S, into thermostated beaker, B, containing distilled water and stirred with a magnetic stirrer, M. The desorbing solution is pumped at a constant rate through the peristaltic pump, P, directly into a thermostated flow-through spectrophotometer cell, C, back into the beaker. M_t , the amount desorbed at time, t , is estimated from the observed absorbance, A_t , of the desorbing solution. (For desorption into 60 ml, suitable absorbance values have been obtained using 5–25 mg wet weight of stratum corneum.)

Equation (4) was evaluated satisfactorily using four exponential terms and additional terms gave no improvement. A polynomial was used to evaluate the error function series and the summation term in equation (5) was evaluated until the change in its value was less than 1×10^{-5} , which is insignificant compared with $1/\pi^4$. A typical regression curve for a set of data is shown in Fig. 2. Parameter estimates are independent of whether equation (4) or (5) is used.

The solute membrane-solution partition coefficient (K_m) may be evaluated from the expression

$$K_m = \frac{(M_\infty - M_i)}{W_m C_s}$$

where W_m is the weight of the membrane, C_s is the concentration of the solute in the solution with which the membrane was equilibrated and $(M_\infty - M_i)$ is the amount of solute desorbed. Some stratum corneum-water partition coefficients are shown in Table 1.

A Collander-type plot¹² of the stratum corneum-water partition coefficient (K_m) against the octanol water-partition coefficient (K_o) shows good correlation (Fig. 3). The values of D/h^2 are indicative of the diffusivities of solutes in a membrane and those for several phenols are listed in Table 1. Good reproducibility of K_m and D/h^2 was found on repeated use of the same membrane, the use of samples of stratum corneum taken from the same area of a cadaver, and initial equilibration of the membrane with different concentrations of solute.

Fig. 2 Typical regression curve for observed absorbance against time following desorption of a solute (*p*-cresol) from hydrated stratum corneum. Correlation coefficient = 0.998; $(D/h^2) \times 10^3 = 1.46 \pm 0.08 \text{ s}^{-1}$; $(A_\infty - A_i) = 0.117 \pm 0.003$; $A_i = 0.010 \pm 0.001$; $\tau = 7.15 \pm 0.15 \text{ s}$; sum squared observations = 0.264; sum of squared deviations (SS) $\times 10^3 = 0.500$.

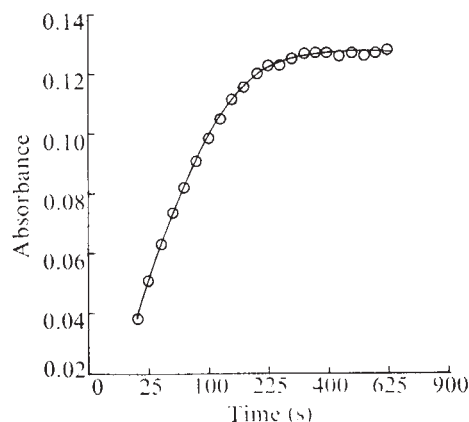


Table 1 Desorption data for human stratum corneum

Solute	$K_m \pm \text{s.d.}$	$D/h^2 \pm \text{s.d.}$ ($\text{s}^{-1} \times 10^3$)	Regression correlation coefficient
Benzyl alcohol	5.0 ± 0.2	0.88 ± 0.04	0.997
Phenylethyl alcohol	5.4 ± 0.3	1.01 ± 0.09	0.992
Phenol	5.4 ± 0.2	0.98 ± 0.05	0.996
<i>p</i> -Cresol	10.3 ± 0.2	1.46 ± 0.08	0.998
<i>m</i> -Cresol	10.4 ± 0.3	1.30 ± 0.07	0.995
<i>o</i> -Cresol	10.5 ± 0.2	1.21 ± 0.08	0.994
<i>p</i> -Bromophenol	27.7 ± 0.4	1.10 ± 0.06	0.997

The wet weight of stratum corneum (separated from other epidermal layers¹⁴) may be estimated by a dehydration technique¹⁵. Combination of D/h^2 , h , and K_m may be used to estimate P using equation (1).

This technique seems to be applicable to studies of drug absorption, pharmaceutical formulation and membrane transport. In the study of drug administration and disposition, the diffusivity and partition behaviour of solutes in various mammalian tissues are of interest.

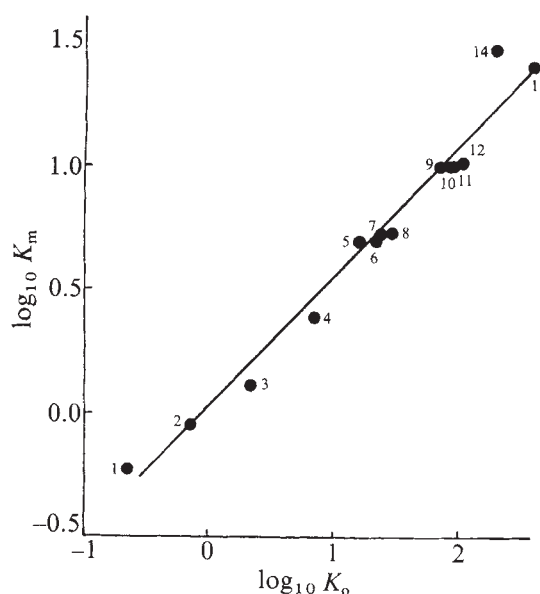


Fig. 3 Collander-type plot of stratum corneum-water partition coefficient (K_m) against octanol-water partition coefficient (K_o)^{3,13} for some non-electrolytes. Compounds are referred to as follows: 1, methanol; 2, ethanol; 3, propanol; 4, butanol; 5, benzyl alcohol; 6, pentanol; 7, phenylethyl alcohol; 8, phenol; 9, hexanol; 10, *p*-cresol; 11, *m*-cresol; 12, *o*-cresol; 13, *p*-bromophenol; 14, heptanol. (Stratum corneum-water partition coefficients are from Table 1 and ref. 4.)

Corneal epithelium (50 μm), stratum corneum (10 μm) and dermis (1,000 μm) are of suitable thickness for evaluation by this technique. For very thin membranes, the technique would have only limited application because desorption would occur rapidly. Since spectroscopy, radiotracer methods, conductometry and so on are suitable analytical methods for this technique, a wide range of solutes may be investigated.

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Macrophages on intrauterine contraceptive devices produce prostaglandins

THE mechanisms whereby intrauterine contraceptive devices (IUDs) exert their anti-fertility effects remains unestablished¹. The involvement of prostaglandins (PGs) is being investigated²⁻⁵, although alternative mechanisms have been proposed (for example, phagocytic and other actions of macrophages^{6,7}). Although the relative contributions of macrophages and PGs to the anti-fertility actions of IUDs remains uncertain, it is clear that introduction of the IUD into the endometrial cavity results in an infiltration by neutrophils and macrophages⁸ as well as increased PG levels in uterine venous blood in animals^{9,10}. While investigating the role of mediators of chronic inflammation we have demonstrated that the macrophage is a potent source of PGE₂¹¹, and since the macrophage is the predominant cell adhering to the IUD⁶ we have considered the possibility that such cells act as a source of PGs.

IUDs routinely removed at out-patient clinics were incubated in 10-20 ml Eagle's MEM supplemented with 10% heat-inactivated foetal calf serum for 24 h at 37 °C in an atmosphere of 5% CO₂ in air. After incubation, supernatants were centrifuged and stored at -20 °C for PG assay. Cells were removed from the devices by treatment with 0.01% trypsin for 5 min at 37 °C, and total cells counted with cell viability established by dye exclusion. Differential cell counts were made on cell smears stained with Toluidine blue, Papanicolaou or Giemsa stains. PG concentrations were determined by radioimmunoassay¹² using sheep antibodies to PGE₂/bovine serum albumin and to PGF_{2 α} /bovine serum albumin. Anti-PGE₂ antiserum did not distinguish PGE₁ from PGE₂ (cross reaction 100%); the relative cross reactivities of other PG groups, based on the amount of unlabelled PG required to displace 50% antibody-bound label, were PGF_{2 α} (13%), PGA₂ (1.3%) and PGB₂ (< 0.6%). Anti-PGF_{2 α} antiserum (cross reaction of PGF_{2 α} 100%) also cross reacted with PGF_{1 α} (27%), but only slightly with PGE₂ (< 0.13%), PGA₂ (< 0.013%) and PGB₂ (< 0.016%). Results were expressed as PGE₂ or PGF_{2 α} equivalents. Seventeen IUDs have been examined so far including eleven of the type Gravigard Copper 7 (Searle, UK), four Lippes loops (Ortho, USA), and two Dalkon shields (Robins, USA). In all cases significant PG activity has been detected in culture fluids collected at 24 h (Table 1). The time course of PG production was determined for several devices

Table 1 PG production by IUDs in *in vitro* culture

IUD (n)	Total WBC ($\times 10^7$) mean $\pm$ s.e.m.	Prostaglandin-like activity in culture fluid (ng PG eq. per 10^7 cells per 24 h) mean $\pm$ s.e.m.	
		PGE ₂	PGF _{2α}
Copper 7 (11)	2.25 ± 0.67	190 ± 52	464 ± 110
Lippes loop (4)	2.00 ± 0.78	161 ± 107	287 ± 198
Dalkon shield (2)	7.50 ± 2.9	92 ± 38	249 ± 37

IUDs cultured in Eagle's MEM and 10% foetal calf serum for 24 h at 37 °C in a 5% CO₂ in air atmosphere. Cells removed by centrifugation and counted. Supernatants stored for assay. 50- μl volumes assayed for PGE₂ and PGF_{2 α} activities by radioimmunoassay. WBC, White blood cells; n, number of IUDs.

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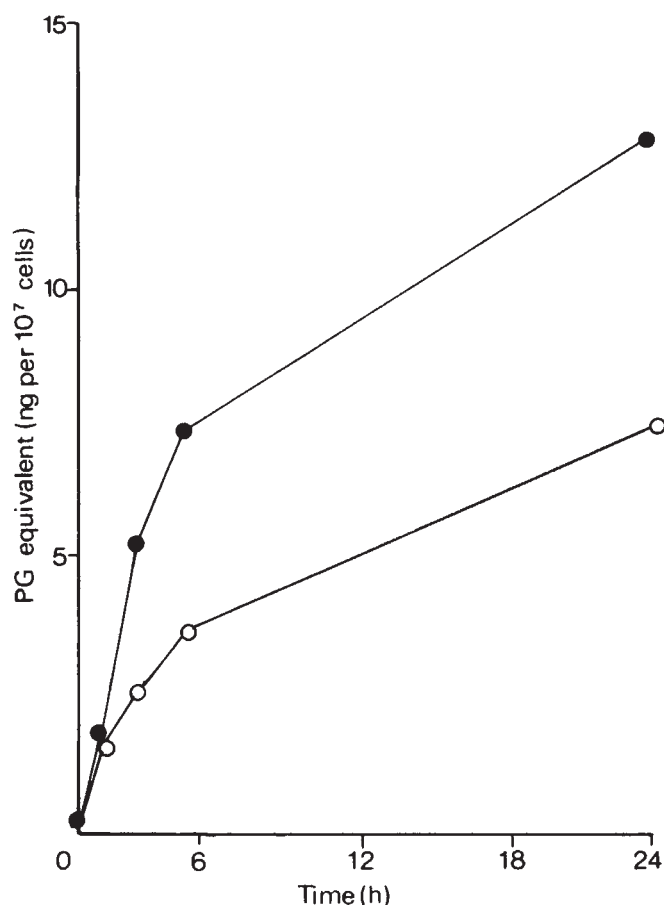


Fig. 1 Time course of prostaglandin (PG) generation for a Copper 7 IUD. The ordinate depicts the concentration of PGE₂ (○) and PGF_{2α} (●) expressed as ng per 10⁷ cells as determined by radioimmunoassay.

and Fig. 1 shows a time course for PGE₂ and PGF_{2α} production by a Copper 7. In general, the PG production correlated with the total cell number on the IUD, and production of PGF rather than PGE predominated in 16 of the 17 devices examined. This contrasts with the guinea pig macrophage where the PGFs constitute less than 10% of the PG activity¹¹ and may relate to the close proximity of macrophages with the copper component of the Copper 7 device, since copper modifies PG production in other situations^{13,14}, or to the regulatory actions of steroid hormones on macrophages¹⁵. Cells identified morphologically as macrophages were the principal cell type on each device. Substantial numbers of neutrophils were observed on some devices but were not associated with enhanced PG production, and in view of the comparative ineffectiveness of neutrophils in PG generation compared with macrophages^{11,16-18}, it seems unlikely that the neutrophil is the source of the PG activity associated with IUDs.

These observations relate two lines of evidence concerning the suggested mode of action of IUDs by showing that the macrophages adherent to IUDs are a likely source of the increased PG concentrations associated with these contraceptive devices. Because the macrophage is such a potent source of prostaglandin, it is worth considering this cell as a source of uterine prostaglandins. Macrophages show cyclical changes in activity in response to altered circulating steroid levels during the oestrous cycle¹⁵, and thus may provide a mechanism whereby the changes in steroid levels can determine the variations in PG production by the uterus during the oestrous cycle¹⁹.

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Cytocidal and cytostatic effects of activated peritoneal leukocytes

MACROPHAGES seem to be of importance in both immunologically specific and nonspecific resistance to neoplastic cells¹⁻¹⁰. It has been proposed¹¹⁻¹³ that activated macrophages are significant effectors in the elimination of tumour cells at sites of delayed hypersensitivity reactions induced by chemical haptens, microbial antigens or lymphokines¹³⁻¹⁸. Immunologically specific elimination of neoplastic cells by macrophages may be mediated by tumour-specific antibodies^{7,10,19,20}. Such antibodies may also confer specificity to macrophages activated by nonspecific stimulation. Besides specificity which may be imparted by antibodies, activated macrophages seem to possess an inherent recognition mechanism which permits selective cytotoxicity towards certain categories of cells, including tumour cells^{11,21-23}.

Although it seems that activated macrophages are cytotoxic towards neoplastic cells^{8,9,11,12,21-23}, there are seemingly conflicting reports on the effects of activated macrophages on cells established from normal tissues. In a number of *in vitro* studies^{11,21-23} insignificant or no effects of activated mouse or rat peritoneal macrophages were noted on fibroblasts of normal adult or embryonic tissue origin. Normal mouse embryonic fibroblasts have, however, been reported to be destroyed by peritoneal leukocytes of nonspecifically stimulated mice²⁴. Our present results offer a possible reconciliation of these differing observations. Rat embryonic cells of a low *in vitro* passage number and a concomitant high rate of replication were found to be susceptible to cytotoxic effects of peritoneal leukocytes of nonspecifically stimulated syngeneic rats. In contrast, embryonic cells of a higher *in vitro* passage number and a lower rate of replication were relatively resistant to such leukocyte preparations.

Rats of the inbred Fischer strain were used. Cells of whole rat embryos close to term were stored in liquid nitrogen. In any one experiment the embryo cells were from the same batch in the second (FE₂) or the sixth (FE₆) *in vitro* passage. Sarcomas were induced by injection of newborn rats with polyoma virus. Cells of these tumours (PYT) were established *in vitro*. Peritoneal leukocytes were collected by intraperitoneal lavage of rats which 48 h previously had received an intraperitoneal injection of 50 µg PPD (purified protein derivative of tuberculin). The differential counts of the peritoneal cells were: large mononuclear cells, 65-75%; small lymphocytes, 10-15%; neutrophils,

Table 1 Effect of challenge of PYT, FE₂ and FE₆ cells with peritoneal leukocytes from PPD-stimulated Fischer rats

Culture combination	Target cells per dish (× 10 ³) at time 0	at 48 h	Unchallenged cultures (%)	Growth index
PYT+M	241; 217	1,747; 1,652	100	7.5
PYT+SP		75; 62	4; 4	
FE ₆ +M	115; 105	188; 182	100	1.7
FE ₆ +SP		144; 141	77; 76	
FE ₂ +M	140; 157	552; 545	100	3.7
FE ₂ +SP		138; 136	25; 25	

Duplicate cultures of PYT or rat embryo cells in 2nd or 6th *in vitro* passage were challenged with peritoneal leukocytes (SP) of a PPD-stimulated rat. Companion cultures received medium (M) only. After 48 h the cells were collected and the target cells counted electronically. The growth index is the ratio between cell counts at 48 h and time 0 of unchallenged cultures.

4–12%; eosinophils, 4–8%, mast cells, 4–8%. Cytotoxicity of the peritoneal leukocytes towards the various target cells was tested as follows. Target cells were seeded in 35-mm round plastic dishes 24 h before use and incubated at 37 °C in a water-saturated atmosphere containing 5% CO₂. On the day of initiation of an experiment (time 0) the culture fluid was removed and replaced with 3 ml of either fresh medium or a suspension of 2 × 10⁶ peritoneal leukocytes. After a further 48-h incubation the cells of duplicate cultures of the various experimental groups were collected and the target cells counted electronically. These procedures, as well as the basis for the use of PPD stimulation of the peritoneal leukocyte donors, have been described previously^{23, 25}.

In a series of four experiments, cultures of PYT, FE₆ and FE₂ cells were challenged with peritoneal leukocytes from PPD-stimulated Fischer rats. A set of companion cultures remained unchallenged. The data from one such experiment are presented in Table 1; comparable figures were obtained in the other three experiments. The peritoneal leukocytes substantially reduced the numbers of the sarcoma cells (Table 1). The final relative counts of these cells were of the order of 4% of the unchallenged companion cultures and their absolute numbers were significantly lower than the time zero counts. In contrast to the sarcoma cells, FE₆ embryo cells increased in numbers when cultured with the peritoneal cells, reaching 76–77% of the cell numbers of the unchallenged controls. The numbers of the FE₂ embryo cells, on the other hand, remained essentially stationary when challenged with the same peritoneal leukocyte preparation. These findings suggest that susceptibility to the cytotoxicity of activated peritoneal macrophages is not solely an attribute of neoplastic cells. The effect of the activated peritoneal leukocytes on the low passage embryonic cells, however, had more the character of growth inhibition than the frankly cytotoxic effect on the sarcoma cells.

Previous observations^{11, 21–23} have indicated that susceptibility or resistance of cells to the cytotoxicity of activated peritoneal leukocytes is not likely to be related to recognition of target cell antigenic determinants, nor is it a consequence of non-specific culture conditions, such as crowding of cells or metabolic depletion. Table 1, together with other findings^{23, 26}, suggests an association between growth rate of normal embryo cells and susceptibility to the cytotoxicity of nonspecifically activated peritoneal leukocytes. Of possible relevance to this association is the agglutinability of non-transformed 3T3 mouse cells by plant lectins only when the cells were in mitosis²⁷. The same 3T3 cells after transformation by polyoma virus, however, were agglutinated by the lectins at any time, irrespective of their position in the growth cycle. It seems therefore that transformed cells continuously exhibit surface properties which normal cells only exhibit when they are in mitosis. Such or similar properties are possibly also reflected in the differential cytotoxic effects reported here. The mechanism of the cytotoxic and the cytostatic effects of activated macrophages may not be fundamentally different. In both situations

destruction of susceptible target cells is likely to take place. The final outcome of macrophage interactions with normal cells in terms of changes in target cell numbers in given culture conditions with respect to macrophage and target cell densities, however, may depend on the relative numbers of target cells in mitosis.

An alternative interpretation of the difference in susceptibility of the high and low passage embryonic cells is selection of a relatively resistant population during *in vitro* cultivation. When initially prepared, embryo cells represent a very heterogeneous population. Certain segments of this population may be susceptible, whereas others may be resistant to the cytotoxicity of activated peritoneal leukocytes. After a number of passages *in vitro* a change in the proportions of these segments may take place resulting in a population with a higher overall resistance.

Neoplastic cells may have certain properties in common with embryonic cells. An additional consideration, therefore, is that the apparent difference in susceptibility of embryo cells of low and high *in vitro* passage number could be attributable to cellular differentiation during cultivation *in vitro*. At the time of isolation from the embryos, the cells may exhibit certain surface properties which are lost during growth *in vitro* as a sequel to differentiation. Although macrophages are present in noticeable numbers in some embryonic tissues at certain stages of differentiation²⁸ a possible relationship of the observations reported here to a specific function of macrophages in embryonic development is at present conjectural.

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Partial characterisation of Ia antigens on murine thymocytes

GENES controlling specific immune responsiveness at the level of activation of the thymus derived (T) lymphocyte, and collaboration of these cells with lymphocytes derived from the bursal equivalent (B lymphocytes), have been shown to be linked to the major histocompatibility complex (MHC) in many species¹. The mechanism of action of these genes is not understood, and it has been postulated that they represent structural genes for a non-immunoglobulin receptor for antigen on T lymphocytes².

In the mouse, the immune response (Ir) genes linked to the MHC have been mapped to the I region of the H-2 complex³. The I region has recently been shown to be associated with antigenic molecules (Ia antigens) expressed predominantly on B lymphocytes, but also on several non-lymphoid populations. The Ia antigens might represent products of the Ir genes. Paradoxically, in view of the strong evidence for the expression of the MHC-linked Ir genes in T cells⁴, whether or not Ia antigens are expressed on T cells has been a matter of some controversy⁴⁻¹⁰. Because of the continuing debate on the nature of the T-cell receptor^{11,12}, the question of the expression of Ia antigens on T lymphocytes is of considerable importance. Previous work from this and other laboratories has indicated that Ia antigens can be detected on T lymphocytes^{8,10} and T lymphoma cells⁹. Ia antigens from several cell sources have also been partially characterised by immunochemical techniques^{8,13,14}, although so far attempts to isolate Ia antigens from thymocytes have proved unsuccessful. In this report, we demonstrate the specific immunological precipitation of Ia antigens from extracts of surface radioiodinated thymocytes.

The outer membranes of thymocytes from A. TL mice were radioiodinated by the lactoperoxidase technique¹⁵ and extracted with the non-ionic detergent Nonidet P-40 (NP-40). The cell extract was then held with A.TH anti-A.TL (anti-I^a; experimental) serum or A.TL anti-A.TH (anti-I^a; control) serum, followed by rabbit anti-mouse IgG serum. The resulting precipitates were centrifuged, washed, and analysed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) and mercaptoethanol¹⁶.

As shown in Fig. 1, radioiodinated surface immunoglobulin was not detected in these conditions. It has previously been shown that NP-40 extraction does not allow clear demonstration of immunoglobulin on murine thymocyte membranes, although it is very efficient in extraction of immunoglobulin from B lymphocytes¹⁷. In our first experiments, a large peak of radioactivity migrating a little ahead of the γ chain in both control and experimental precipitates was found. It was precipitable by IgG-anti-IgG, IgM-anti-IgM and fowl IgG-anti-fowl IgG complexes, and was invariably present in comparable amounts in control and experimental precipitates. This peak has been observed elsewhere^{18,19}, and was considered to be a component which binds to antigen-antibody complexes. In subsequent experiments, this component was depleted by a factor of approximately five by preliminary precipitation with fowl IgG and rabbit anti-fowl IgG (legend to Fig. 1). In addition, a small but unequivocal peak of radioactivity migrating just behind the light chain standard was consistently observed in the experimental (A.TH anti-A.TL) but not in the control (A.TL anti-A.TH) precipitates. Identical results were obtained in three independent experiments, and the peak was also clearly visible in autoradiographs. This peak corresponded to a molecular weight of approximately 30,000, although determination of molecular weight by polyacrylamide gel electrophoresis must be made with some caution, as carbohydrate differences considerably influence mobility²⁰. A third peak, corresponding to an R_f of approximately 0.05, was seen in some experiments. It was rather variable in size, but was

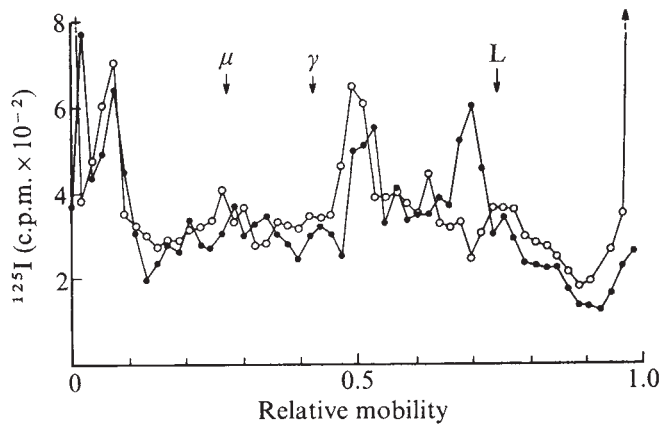


Fig. 1 Thymuses from 6-week-old female A.TL mice were removed, taking care to avoid removal of the parathyroid lymph nodes, and teased gently through a fine sieve into phosphate-buffered saline (PBS). Dead cells and clumps were removed by the method of von Boehmer and Shortman²¹. Cells were surface radioiodinated with ¹²⁵I as follows. To five plastic centrifuge tubes, each containing 10⁷ cells in 30 μ l PBS, was added 10 μ l lactoperoxidase (0.25 mgml⁻¹), 5 μ l carrier free ¹²⁵I sodium iodide (0.05 mM, 100 μ Ci μ l⁻¹), and 10 μ l 8.8 mM H₂O₂. Cells were held at 30 °C for 5 min, pooled, and washed once with 5 ml PBS. They were then extracted with 1% NP-40 in PBS (3.5 ml) at room temperature for 1 h, centrifuged, and the supernatant dialysed against PBS for 4 h at 4 °C. The extract was then centrifuged at 10,000g for 30 min, and the supernatant divided into 200- μ l aliquots, to which were added 100 μ l rabbit anti-fowl IgG (absorbed against mouse immunoglobulin) and 100 μ l of 0.1 mg ml⁻¹ fowl IgG. The mixture was held at 37 °C for 1 h, and 4 °C overnight. It was then centrifuged at 10,000g for 20 min, and the precipitates discarded. To each tube was added 2 μ l A.TH anti-A.TL serum, or A.TL anti-A.TH serum prepared as previously described⁶. Both antisera were extensively absorbed for autoantibodies before use. The extracts were incubated at 37 °C for 1 h, and then an appropriate amount of rabbit anti-mouse IgG serum added quantitatively to precipitate the mouse IgG. A further incubation was performed at 37 °C, and the tubes left at 4 °C overnight. The precipitates were washed four times, and then boiled in a buffer solution containing 5% mercaptoethanol and 3% SDS, and analysed by discontinuous polyacrylamide gel electrophoresis as described by Laemmli and Favre¹⁶. The total concentration of polyacrylamide was 10%, and the concentration of bisacrylamide was 0.25%. Gels were 10 cm long and 5 mm in diameter. The precipitated material from 5 $\times$ 10⁷ cells was ultimately distributed among four gels (two control and two experimental), each gel thus representing material from 1.25 $\times$ 10⁷ cells. Gels were sliced transversely into 2-mm sections and counted in a Packard gamma counter, or sliced longitudinally and processed for autoradiography. Calibration of molecular weights was done by running parallel gel electrophoresis of proteins of known molecular weights. ●, A.TH anti-A.TL serum; ○, A.TL anti-A.TH serum.

always of similar height in experimental and control precipitates. This peak may represent high molecular weight aggregates.

It is unlikely that the 30,000 molecular weight peak is due to the presence of small numbers of B lymphocytes in the thymocyte cell suspension. The fraction of A.TL thymus cells with high density surface immunoglobulin was found to be less than 0.5%, as assessed by autoradiography using polyspecific radioiodinated anti IgM serum, and we are unable to detect Ia extracted from this number (6 $\times$ 10⁴) of B lymphocytes. Further, it has been demonstrated that the TCA precipitable radioactivity incorporated into NP-40 extractable immunoglobulin on B cells following surface iodination is considerably more than that incorporated into B cell Ia antigens¹⁴, yet no radioactivity corresponding in mobility to immunoglobulin chains was seen (Fig. 1). Thus, the relative amounts of Ia and immunoglobulin do not support the hypothesis that the detected Ia was obtained from B cells.

There are several possible reasons for our ability to extract and identify Ia from thymocytes. First, and probably of prime importance, is the titre and specificity of the anti-Ia serum used. The A.TH anti-A.TL serum was of very high titre (1 : 2,000

against spleen cells by semi-microcytotoxicity and 1 : 10,000 by microcytotoxicity tests using rabbit complement), and has been shown lightly but specifically to label 10–20% of thymocytes in autoradiographic experiments⁸. Fathman *et al.* (C. G. Fathman, J. L. Cone, S. O. Sharrow, H. Tyrer, and D. H. Sachs, personal communication) have also shown, using the fluorescence activated cell sorter, that a subpopulation of thymocytes bears Ia antigens in small amounts. Many anti-Ia sera, usually of much lower titre, only react with B cells⁴. Second, labelling by surface radioiodination, rather than biosynthesis using radiolabelled amino acids, allows a far better signal to noise ratio, since radioactivity is confined to the area of interest. Third, optimal iodination conditions and excellent cell viability, both before and after iodination, were found to be essential for good results.

Our results are consistent with the serological data indicating that Ia antigens are present in large amounts on B lymphocytes and in much smaller amounts on T lymphocytes^{8,10}. The sera used in our studies potentially recognise the products of all I subregions. In view of the increasing evidence for heterogeneity of I region products, future work must concentrate on the use of sera of more restricted specificity, and attempt to understand the possible relationships between the Ia antigens and products of the immune response genes.

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Isolation and properties of a murine spleen cell Fc receptor

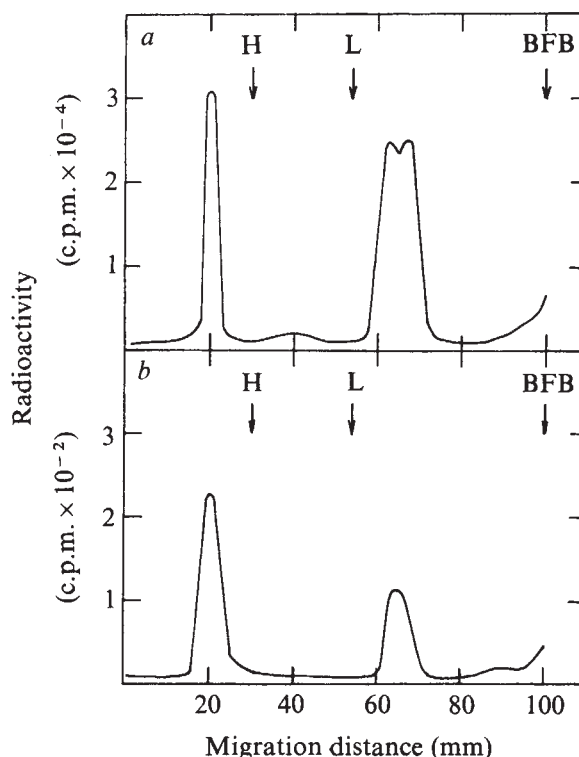
MURINE and human bone marrow-derived (B) lymphocytes have a cell surface-bound receptor for antibody complexed to antigen. Heat-aggregated immunoglobulin complexes will also bind to this receptor, and the binding is specifically mediated by means of the Fc portion of the IgG molecule^{1–3}. So far, the only function ascribed to the Fc receptor is in the antibody-dependent lymphocyte-mediated cytotoxicity reaction³. A recent report has indicated, however, that the Fc receptor is identical to or closely associated with the Ia antigens on the B-cell membrane⁴. The Ia antigens are alloantigens coded for by the immune response region of the major histocompatibility

complex⁵ and if indeed these antigens and the Fc receptor are identical, this would reveal a role for the receptor in several aspects of the immune response⁶.

To gain insight into the possible relationship between the Fc receptor and the Ia antigens it seems mandatory to isolate and characterise chemically these molecules. As a first step towards this goal we have isolated an Fc receptor from mouse spleen cells and here report some of its properties.

Crude membrane fractions were isolated from 400 A/Sn mouse spleens. The membrane fraction was suspended in 0.02 M Tris-HCl buffer, pH 7.4, containing 80 mM EDTA and 50 mM β -mercaptoethanol at a final protein concentration of 10 mg ml⁻¹. Soluble macromolecules were recovered after centrifugation of the incubation mixture at 100,000g for 60 min. The supernatant was subsequently subjected to affinity chromatography on a column of Sepharose 4B to which heat-aggregated human IgG had been covalently attached. The column was eluted as described⁷. The eluted macromolecules were labelled with ¹²⁵I and subjected to sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis⁷ together with suitable marker proteins of known molecular weight. Figure 1 depicts a typical result of an electrophoretic separation in SDS of ¹²⁵I-labelled macromolecules with affinity for heat-aggregated IgG. Three types of polypeptide chains with apparent molecular weight of approximately 65,000, 18,000 and 15,000 were clearly distinguishable. Figure 1 also demonstrates that the 65,000-dalton component as well as one (or both) of the two smaller polypeptide chains contained sialic acid as evidenced by radioactive labelling with ³H-sodium borohydride⁸. The three ¹²⁵I-labelled polypeptide chains were extensively reduced and alkylated in 6 M guanidine hydrochloride and subjected to gel chromatography on a column of Sepharose 6B equilibrated with the same solvent. The elution

Fig. 1 SDS-polyacrylamide gel electrophoresis of ¹²⁵I-labelled (a) and ³H-sodium borohydride-treated (b) highly purified mouse spleen cell Fc receptor. The macromolecules were passed over a Sepharose 4B-coupled heat aggregated IgG column. Material attached to the column was eluted with 0.05 M sodium citrate buffer, pH 3.0, containing 0.5 M NaCl. The recovered protein, labelled with ¹²⁵I or ³H, was introduced into sialic acid residues. H and L denote the migration positions of marker heavy and light chains of IgG. The marker dye was bromophenol blue (BFB).



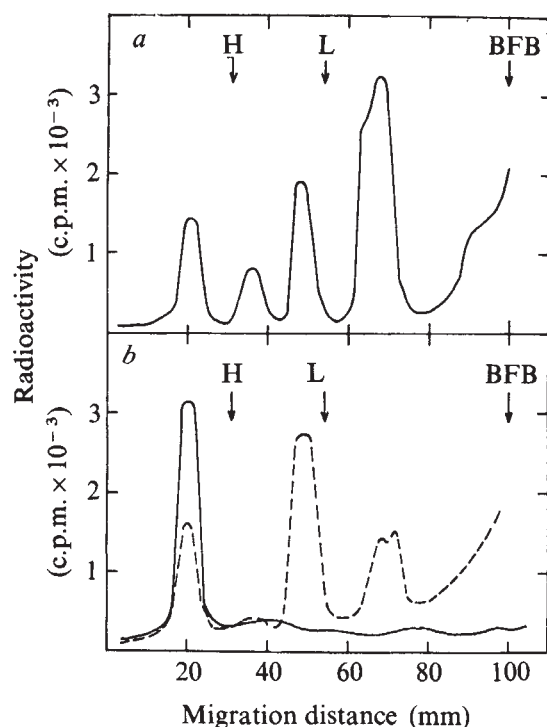


Fig. 2 SDS-polyacrylamide gel electrophoresis of ^{125}I -labelled highly purified mouse spleen cell Fc receptor. *a*, The material was the same as shown in Fig. 1 but the electrophoresis was performed after 2 d incubation at 4°C . *b*, The 65,000-dalton component after thermolysin. The ^{125}I -labelled 65,000-dalton component was mixed with bovine IgG and digested with thermolysin (20% w/w) for 10 min at 37°C . —, Material before proteolysis; — — —, after thermolysin digestion.

positions of the ^{125}I -labelled molecules corresponded to apparent molecular weights of 65,000, 14,000 and 13,000, respectively. The higher molecular weight values obtained by SDS-electrophoresis are consistent with the presence of carbohydrate in the Fc-receptor proteins. This strongly suggests that each component represents a single polypeptide chain.

The specificity and efficiency of the isolation procedure was investigated by incubating ^{125}I -labelled samples of the crude membrane protein and of the breakthrough material from the affinity chromatography column with antigen-antibody complexes. The antigen-antibody complexes were formed from bovine serum albumin (BSA) and a heat-inactivated rabbit antiserum against BSA. After 30 min of incubation, precipitates were collected by centrifugation, extensively washed and subjected to SDS-polyacrylamide gel electrophoresis. The electrophoreses revealed that the starting material contained the same three components which had been isolated by affinity chromatography, but the smaller polypeptide chains were now the dominant components. The absorption on to the affinity chromatography column was very efficient since the breakthrough material recovered from the column contained negligible amounts of protein reacting with the antigen-antibody complexes.

Similar experiments showed that the isolated Fc-receptor components retained their affinity for antigen-antibody complexes even after desorption from the affinity chromatography column.

The three polypeptide chains were encountered in all highly purified preparations. On standing at 4°C for a few days additional components arose, presumably because of the presence of proteolytic activity in the preparations. Figure 2*a* depicts an SDS-polyacrylamide gel electrophoresis of the same material as shown in Fig. 1, but after 2 d at 4°C . It can be seen that in addition to the 65,000, 18,000 and 15,000-dalton components, new material appeared in the apparent molecular weight positions 30,000 and 45,000, respectively. This result

suggested that the 18,000 and 15,000-dalton components were degradation products of the 65,000-dalton molecule. That this indeed is the case was demonstrated by subjecting the isolated 65,000-dalton component to thermolysin digestion. Figure 2*b* shows that such treatment gave rise to three additional polypeptide chains. The main component had an apparent molecular weight on SDS-polyacrylamide gel electrophoresis of about 30,000, whereas the two smaller components had molecular weights of 18,000 and 15,000, respectively. Solubilisation of membrane macromolecules by treatment with NP-40 or by papain digestion usually gave Fc-receptor components with apparent molecular weights of about 30,000 and 15,000 as estimated on SDS-polyacrylamide gel electrophoresis. A common origin for the 65,000, 18,000 and 15,000-dalton components was also suggested by the immunological cross reactivity between the three polypeptide chains (L.K., L.R. and P.A.P., unpublished).

Rabbit antisera were raised separately against the 65,000-dalton material and the mixture of the 18,000 and 15,000-dalton components. Table 1 shows that such antisera reacted exclusively with B lymphocytes. That the antisera bound to the B-cell Fc receptor was documented in a separate experiment. Aggregated IgG was labelled with fluorescein isothiocyanate³ (FITC) and allowed to bind to spleen cells treated with various antibody IgG and (Fab')₂ fractions. Table 2 shows that intact antibodies of various specificities all impeded the binding of aggregated IgG provided they bound to the B-cell surface. This result is in agreement with previous findings⁹. When (Fab')₂ fragments, lacking the Fc portion, of these antisera were used, however, only those directed against the isolated 65,000-dalton and the 18,000–15,000-dalton components mixture were effective (Table 2). The (Fab')₂ fragments against H-2^k were also effective in reducing the number of cells containing aggregated IgG. This antiserum retains its ability to abolish binding of aggregated IgG to B cells even after all measurable anti-H-2 alloantigen activity has been absorbed out on thymocytes of the relevant antigen type. It seems reasonable to conclude that the antiserum contains anti-Ia antibodies since similar results were obtained with a specific anti-Ia serum (Table 2)^{4,9}.

If the Fc receptor and the Ia antigens are physically associated

Table 1 Cell binding of antibodies against the 65,000-dalton polypeptide chain and against the mixture of the 18,000 and 15,000-dalton components

Cell source	Treatment*	Antiserum	Stained cells (%)
Spleen	—	65,000	44 ± 6
Spleen	—	18–15,000	42 ± 8
Spleen	anti-Thy1.2	65,000	86 ± 4
Spleen	anti-Thy1.2	18–15,000	81 ± 11
Lymph nodes	—	65,000	22 ± 2
Lymph nodes	—	18–15,000	20 ± 3
Spleen	—	Ig	42 ± 5
Lymph nodes	—	Ig	16 ± 6

The cells obtained from C₃H/He mice were adjusted to a final concentration of 5×10^7 cells per ml in Hanks' minimal essential medium (MEM) containing 2% bovine serum albumin. Samples of 50 μl were incubated with 5 μl antiserum of the appropriate specificity for 30 min at 4°C . The cells were washed twice with the medium, resuspended in 50 μl of fresh medium, and incubated with 5 μl of FITC-conjugated goat anti rabbit IgG (14 mg ml⁻¹) for another 30 min at 4°C . At the end of the incubation period, the cells were washed three times with the medium and examined in a Leitz Ortholux microscope equipped with a HBO 200-W lamp. Ploem-type vertical illuminator and immersion objectives were used. In each experiment 200 cells were scored for cell surface fluorescence.

*To 10^8 spleen lymphocytes 0.3 ml of an anti-Thy1.2 antiserum was added and incubation was allowed to proceed for 30 min at 4°C . The cells were washed twice in Hanks' MEM and resuspended in 5 ml of a 1/10-dilution of fresh rabbit serum absorbed on agarose. The medium also contained DNase (10 μg ml⁻¹). Incubation was at 37°C for 30 min. Cell debris was removed by extensive washing of the cells in Hanks' MEM, and the incubation with antibodies was carried out as described above.

Table 2 Effect of antibodies and antibody (Fab')₂ fragments of various specificity on the binding of aggregated IgG to murine spleen cells

Antibody specificity*	Pretreatment of cells with IgG (Fab') ₂ % Stained cells	
NRS	42 ± 4	41 ± 5
RBP	44 ± 6	41 ± 6
65,000	6 ± 1	8 ± 2
18-15,000	8 ± 2	7 ± 1
β ₂ μ	7 ± 3	41 ± 7
H-2K ^k	5 ± 2	6 ± 2
H-2K ^k (abs.)	7 ± 2	6 ± 3
Ia ^k	11 ± 3	8 ± 2

Spleen cells from C₃H/He mice were suspended in Hanks' MEM containing 2% bovine serum albumin at a final concentration of 5×10^7 cells per ml. Samples of 50 μl were incubated with 5 μl of the appropriate IgG or (Fab')₂ fraction (15 mg ml⁻¹). After incubation for 30 min at 4 °C, the cells were washed twice in Hanks' MEM. The cell concentration was again adjusted to 5×10^7 cells per ml and 50 μl of FITC-labelled aggregated human IgG (about 1 mg ml⁻¹ in phosphate-buffered saline containing 2% bovine serum albumin and 2 mM sodium azide) was added. After incubation for 30 min at 4 °C, the cells were washed twice and 200 cells were regularly scored for label. The data are the mean ± s.e.m. of three experiments.

* IgG was isolated from immune sera by a combination of sodium sulphate precipitation and Sephadex G-200 chromatography. (Fab')₂ fragments were prepared from the purified IgG by pepsin digestion. (Fab')₂ fragments were freed from remaining intact antibodies by gel chromatography on columns of Sephadex G-200. NRS denotes gamma globulin from a normal rabbit serum. RBP is a human serum protein. The anti-β₂-microglobulin (β₂μ) antiserum was raised in a rabbit against the human protein. This antiserum cross reacts with the mouse homologue. The anti-H-2K^k was raised in (BALB/c × C57BL/6) F₁ mice against A/Sn spleen cells. Absorption of this antiserum was accomplished with C₃H/He thymocytes. Before absorption the antiserum could kill more than 85% of spleen cells and thymocytes from the appropriate strains. After final absorption the antiserum was not cytotoxic against either cell type.

on the cell surface or indeed identical, antibody-induced redistribution and pinocytosis of the Fc receptor would simultaneously eliminate the Ia antigens from the cell membrane. Table 3 shows that although the Fc receptor could be capped away efficiently, no apparent redistribution of the Ia antigens could be demonstrated by the cytotoxicity technique. In a second set of experiments Ia antigens and the Fc receptor were solubilised from a crude C₃H/He spleen cell membrane fraction. The inhibition of anti-Ia^k induced cytotoxicity measured separately on spleen cells from BALB/c and C₃H/He mice was estimated before and after the Fc receptor had been removed from the other solubilised membrane proteins on an aggregated IgG column. Although more than 80% of the Fc receptor could be eliminated from the bulk of material passing unretarded through the column, the content of Ia antigens was unchanged in this fraction. Furthermore, the isolated Fc receptor did not measurably impede the cytotoxic activity of the anti-Ia^k serum.

Trypsin treatment of spleen cells does not affect the presence of the Fc receptor on B cells³. Accordingly, C₃H/He spleen cells were digested with trypsin as described³ and the amounts of Fc receptor and Ia antigens reacting with the appropriate antisera on the cell surface were quantitatively estimated before and after the proteolytic digestion. Whereas trypsin treatment did not change significantly the amount of antibodies bound to the Fc receptor, more than 80% of the Ia antigens were lost. Thus, our results fail to demonstrate a physical association between the Fc receptor and the Ia antigens.

In conclusion, murine B cells seem to carry a glycoprotein which binds to antigen-antibody complexes. Antiserum against this macromolecule abolishes the binding of aggregated IgG to B lymphocytes, demonstrating that the isolated polypeptide chain constitutes the whole or part of the Fc receptor. Although antibodies against Ia also impede the binding of aggregated IgG to B cells, several attempts to demonstrate the presence of

Ia antigenic determinants on the Fc receptor were unsuccessful. This information, together with the fact that the molecular weight of the Ia antigens is only about half that of the isolated Fc receptor¹⁰⁻¹³, suggests that the Fc receptor is distinct from the Ia antigens. The effect of antisera against Ia antigens to displace aggregated IgG does not seem to depend on a physical linkage between these antigens and the Fc receptor but may merely reflect a situation where the Fc receptor in the cell membrane is located adjacent to the Ia antigens.

Table 3 Relationship between Ia antigens and the Fc receptor on the cell surface

Antibody specificity*	Target cells	Pretreatment of cells	Dead cells (%)
18-15,000	H-2 ^k	NRS	47 ± 4
18-15,000	H-2 ^s	NRS	44 ± 3
Ia ^k	H-2 ^k	NRS	55 ± 9
Ia ^k	H-2 ^s	NRS	6 ± 3
Ia ^s	H-2 ^k	NRS	4 ± 2
Ia ^s	H-2 ^s	NRS	41 ± 6
18-15,000	H-2 ^k	18-15,000	6 ± 4
18-15,000	H-2 ^s	18-15,000	8 ± 3
Ia ^k	H-2 ^k	18-15,000	51 ± 7
Ia ^s	H-2 ^s	18-15,000	44 ± 5

Spleen cells from C₃H/He (H-2^k) or A.SW(H-2^s) mice were suspended in Eagle's MEM with 10% FCS at a final concentration of 20×10^6 cell per ml. Samples of 200 μl were pretreated with 50 μl of antiserum against the Fc receptor or with normal rabbit serum (NRS) for 4 h in a 5% CO₂ atmosphere at 37 °C. After incubation the cells were washed twice in cold Hanks' MEM with 5% FCS. The cell concentration was adjusted to 1×10^6 cells per ml, 25 μl samples were subjected to the cytotoxicity assay with use of the antiserum indicated. Dead cells were stained with Trypan blue. At least 200 cells were examined in each test. The data are the mean ± s.e.m. of three to five experiments.

* The A.TH anti-A.TL (anti-Ia^k) and the A.TL anti-A.TH (anti-Ia^s) antisera were given by Dr G. Hämmerling. All antisera were heat inactivated before use and freed from aggregated IgG by ultracentrifugation.

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Tumour rejection properties of solubilised TSTA from an SV40-induced neoplasm

THE neoplasm mKSA, an SV40-induced sarcoma in BALB/c strain mice has strong, SV40-specific tumour rejection antigens (TSTA). This parental line is also very oncogenic with a tumour dose 50 (TD_{50}) of 10^2 cells. A subline, passaged in monolayer tissue culture (mKSA-TC), has a much reduced tumour-inducing capacity ($TD_{50} = 2 \times 10^6 - 2 \times 10^7$ cells). Of these mKSA-TC cells 1×10^5 will immunise syngeneic hosts against a challenge of 1×10^6 mKSA cells carried in an ascitic form (mKSA-ASC); immunisation against other SV40-induced BALB/c sarcomas (for example, SVA31C14 and E4) has also been achieved, but not against Adj-PC-5 (ref. 1) nor against Meth A (ref. 2), both neoplasms carrying specific TSTAs and syngeneic with BALB/c but induced by chemical carcinogens.

Recent work in our laboratory showed that methods developed for isolation and solubilisation of biologically active H-2, histocompatibility antigens^{3,4} could be adapted to obtain soluble immunogenic TSTA from both RNA and DNA virus-transformed neoplasms⁵⁻⁷. Preliminary results were reported of solubilised TSTA with tumour rejecting activity from the non-virus-producing tumour, mKSA, induced by SV40⁵. We now report biological activity of ten consecutive solubilised preparations prepared from membranes of either the mKSA-TC or mKSA-ASC forms of this neoplasm.

The antigen preparations assayed for tumour rejection are designated CM, crude membrane; CS, crude soluble; F-2 and F-3 fractions prepared from Sephadex G-150 chromatography (the included volumes). Tumour cells ($2-6 \times 10^6$), either from the ascites form or from the cultured subline were collected in Tris-buffered saline, pH 7.4. Cells were washed twice in a solution containing 0.145 M NaCl, 0.005 M Tris-buffer, pH 7.4 and 2×10^{-4} M $MgSO_4$; this solution was adjusted with distilled water to 0.125 M NaCl. The cells were equilibrated in a

cell disruption bomb at 400 pounds inch⁻² N_2 for 30 min and released rapidly to effect disruption of 90% or more of cells. After removal of nuclei and cell debris the supernatant was centrifuged at 55,000g for 90 min, the new supernatant was discarded and the sediment was suspended in 0.05 M Tris, pH 8.4, at approximately 10 mg dry weight per ml. This CM, was digested with papain at a ratio of 1 mg papain to 100 mg protein, at 37 °C in the presence of 0.01 M dithiothreitol; the reaction was stopped after 1 h by adding iodoacetamide to a final concentration of 0.022 M and insoluble sediment removed by centrifugation at 105,000g for 1.5 h. The soluble supernatant was dialysed exhaustively against 0.15 M NaCl-0.01 M Tris, pH 8.2, and then concentrated by ultrafiltration (Amicon, UM2 membranes). The resulting CS material was applied to Sephadex G-150, the eluate collected and a chromatogram obtained by measuring its absorbance at 280 nm. Fractions F-2 and F-3 were collected and used in the *in vivo* assays. Protein concentrations of CM were determined by dry weight and of CS and the F-2 and F-3 fractions by the method of Lowry *et al.*⁸.

As Table 1 shows, all 10 antigen preparations constituting CM, CS or the chromatographed F-2 and F-3 fractions were effective in immunising BALB/c mice against challenge with the syngeneic mKSA sarcoma. mKSA(TC) and mKSA(ASC) were equally effective and thus share the same TSTA in spite of long-continued passage *in vitro* and *in vivo*. Protection was afforded at cell-challenge concentrations far above the TD_{50} of 10^2 cells. The degree of protection was in the range of that provided by a single subcutaneous injection of 10^5 or 10^6 mKSA-TC cells; the immunisation afforded was also rapid and complete. Injections prepared in Freund's complete adjuvant (FCA) were not more immunogenic than those prepared in Tris-buffered saline. A precise dose-response relationship was not observed in those experiments (6a, 6b, 7) in which graded doses of antigen were used for immunisation and graded challenges of mKSA cells were used. This may mean that we were immunising with concentrations of antigen above optimal levels. *In vitro* specific lymphocyte stimulation was also

Table 1 Tumour rejection by membrane antigen preparations of sarcoma mKSA

Preparation no.	Type of preparation (source of cells)	Immunisation schedule*	No. of mKSA cells in challenge	No. of + No. inoculated	(Controls)
1a	CM(ASC)	70 $\mu\text{g} \times 2$	10^4 and 10^{5+}	0/10	(8/10)
1b	CS (ASC)	100 $\mu\text{g} \times 2$		2/10	(8/10)
2	CM(TC)	1,000 $\mu\text{g} \times 2$		0/10	(8/10)
3	F-2 fraction (TC)	60 $\mu\text{g} \times 2$		1/5	(4/10)
4a	F-2 fraction (TC)	25 $\mu\text{g} \times 2$	“ “	4/10	(10/10)
4b	F-3 fraction (TC)	100 $\mu\text{g} \times 2$	“ “	2/10	(10/10)
		50 $\mu\text{g} \times 2$			
		150 $\mu\text{g} \times 2$			
5	CM(ASC)	1,000 $\mu\text{g} \times 2$	“ “	1/9	(9/10)
6a	CM(ASC)	1,000 $\mu\text{g} \times 2$	10^3 , 10^4 , 5×10^5	0/15	(10/15)
		300 $\mu\text{g} \times 2$		0/15	
		100 $\mu\text{g} \times 2$		3/15	
6b	CS (ASC)†§	100 $\mu\text{g} \times 2$	10^3 , 10^4 , 5×10^5	0/15	(10/15)
		30 $\mu\text{g} \times 2$		0/15	
		10 $\mu\text{g} \times 2$		2/10	
7	CS (TC)	100 $\mu\text{g} \times 2$	7×10^3 , 7×10^4	4/10	11/40 (9/10)
		50 $\mu\text{g} \times 2$		3/10	
		25 $\mu\text{g} \times 2$		2/10	
		2.5 $\mu\text{g} \times 2$		2/10	
8	CS (ASC)	See text for results of Winn assay			
9	CS (TC)¶	50 $\mu\text{g} \times 2$	5×10^4 , 1×10^4	0/8	(14/16)
10	CS (ASC)	30 $\mu\text{g} \times 2$	5×10^4	0/5	(6/10)
		10 $\mu\text{g} \times 2$		3/5	

P values for differences between experimental and control groups vary from 0.05 to <0.001.

*BALB/c female mice, 8-12 weeks old, were given subcutaneous injections of antigen, twice at 2-week intervals. In experiments 1 to 5, injections were prepared in an equal volume of CFA; others were prepared in Tris-buffered saline (TBS). Controls received CFA or TBS as appropriate.

† TD_{50} of mKSA (ASC) challenge was 10^2 cells.

‡Specific lymphocyte stimulation (LS) was demonstrated in a microculture LS assay using the papain-solubilised CS preparations 6b and 7 (ref. 9).

§TSTA activity was reflected in growth rates as well as in frequency of takes. For example mean tumour volume of BALB/c mice immunised with preparation 7 (2.5μ g $\times$ 2), 40 d after challenge was 985 mm³ and 3,756 mm³ for controls.

¶Ratio of 1 mg protein to 50 mg protein used for this preparation.

Table 2 Specificity of immune rejection of mKSA sarcoma in syngeneic BALB/c mice

Treatment immunisation	No. with tumour Total no. (r^2)*	Days to death	Challenge tumours
1a = BALB/c spleen CS†	8/8 (29)	42	mKSA 5×10^4 cells
1b = none	7/8 (30.4)	41	mKSA 5×10^4 cells
2a = mKSA CS (preparation 10)	0/5‡ (—)	—	mKSA 5×10^4 cells
2b = none	6/10 (60.2)	39	mKSA 5×10^4 cells
2a = mKSA CS (preparation 10)	10/15 (25)	50	Meth A 5×10^4 cells
2c = none	4/5 (33)	50	Meth A 5×10^4 cells

* r^2 , Radius² of subcutaneously growing tumours in mm at 30 d after transplantation.

†CS, papain solubilised membranes; 50 μ g injected in CFA $\times 2$, 2 weeks apart. Tumour challenge 2 weeks after last immunisation. Controls received CFA only.

‡These mice were rechallenged after 60 d with 1×10^6 mKSA sarcoma cells and remain negative 45 d after challenge.

observed, using preparations 6b and 7, with concentrations of antigen as low as 0.1 μ g per well ($0.5 \mu\text{g ml}^{-1}$)⁹. This assay presumably reflects a cell mediated immune response.

Our soluble F-2 fraction containing immunogenic material chromatographed as we previously observed with H-2 alloantigens derived from lymphoid cells^{6,7}; nonetheless we also observed our F-3 fraction to be immunogenic. This may represent fragments of the TSTA of low molecular weight produced by excessive papain degradation. Further steps in purification of the mKSA TSTA now in progress, however, should clarify this problem.

In addition to the activity of soluble TSTA from mKSA tumour cells in *in vivo* tumour rejection and in *in vitro* lymphocyte stimulation, specific tumour cell neutralisation was observed using the Winn assay. Sensitised spleen cells from BALB/c mice immunised with several concentrations of the (CS) mKSA could neutralise mKSA tumour cells (Fig. 1). Previous work had shown that the host can recognise TSTA on intact mKSA tumour cells and generate effector cells that specifically destroy the tumour¹⁰.

Specificity of immunogenicity was observed in BALB/c

mice made strongly immune to mKSA antigen and challenged with the syngeneic plasma cell tumour Adj-PC5 (ref. 5). Further demonstrations of specificity are shown in Table 2. Papain-solubilised membranes of BALB/c spleen cells did not confer the capacity to reject mKSA tumour nor did the CS preparation of mKSA sarcoma cells immunise against another BALB/c neoplasm, Meth A, that has its own strong TSTA.

Preliminary comparative studies of the properties of TSTA and H-2 have shown that neither antigen bound significantly to a DEAE-ion exchange column in the presence of 0.05 M NaCl at pH 7.2. In contrast, H-2 bound strongly to a con A affinity column, but TSTA did not (O. H., unpublished). The latter observation suggests that TSTA is a different glycoprotein from H-2, but further purification is required before the antigen can be characterised chemically.

These studies demonstrate that the immunogenic capacity of TSTA like H-2 histocompatibility antigens is preserved by papain digestion and by the limited purification procedures described here. Studies of further purification of SV40-specific TSTA are in progress.

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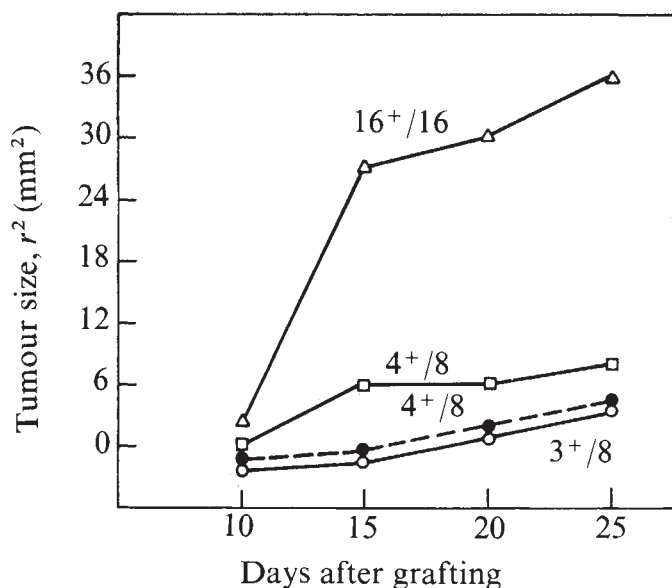


Fig. 1 Results of Winn assay (neutralisation). BALB/c mice were immunised with a single subcutaneous inoculation of preparation 8, CS material in TBS at concentrations of 30 μ g ($\square$), 250 μ g ($\circ$) or 500 μ g ($\bullet$). Two weeks later spleen cells from these immunised groups and from uninoculated controls (Δ) were mixed in a ratio of 100:1 mKSA (ASC) tumour cells and inoculated into BALB/c 8–12-week-old female mice. Sensitised spleen cells from the 250 μ g ($\circ$) immunised group when mixed with Adj-PC5 (BALB/c) tumour cells and inoculated into syngeneic recipients had no effect on frequency or rate of growth. Data not shown. Differences at 25 d, when 50% of controls had died, in both frequency of progressive growth and mean r^2 between experimental and control groups were significant ($P < 0.01$).

Possible role for H-Y antigen in the primary determination of sex

IN mammals, the genetic basis of sex determination and differentiation seems to be simple^{1,2}. A gene or set of genes on the Y chromosome causes the indifferent embryonic gonad to develop as a testis³. Thus an ovary develops in the absence of the Y and a testis in its presence. The testis then secretes testosterone which induces male development of the accessory glands and ducts^{4,5}. The masculinising action of testosterone on its target cells is mediated by the product of a gene on the X chromosome. This product activates all the genes required for manifestation of the male phenotype in response to circulating testosterone⁶. Evidence for this crucial involvement of the

X chromosome in male sexual differentiation comes from a mutation of the relevant gene in the mouse (*Tfm*), resulting in failure to respond to testosterone. A chromosomally XY animal with this mutant gene develops testes, because the Y chromosome is present, but shows no further male differentiation, thus exhibiting the syndrome known as 'testicular feminisation'⁶. This insensitivity to androgen is caused by a mutational deficiency of the nuclear-cytosol androgen-receptor protein not only in mice⁷⁻⁹ but also in man¹⁰. Thus the part that the X chromosome plays in male sexual differentiation has been clarified at the level of an individual gene situated on this chromosome.

The same cannot be said of the part that the Y chromosome plays in inducing testicular development, because no relevant mutation of the presumptive testis-determining gene has been found in any mammalian species, and because we have no clue, therefore, as to the nature of any product specified by this presumptive gene locus. It is clear, however, that the whole Y chromosome is not necessary for this important function. A large part of the mammalian Y chromosome, which as a rule is a relatively minute element, seems to be genetically inert. The observation that an apparent isochromosome of the long arm of the human Y chromosome has no testis-determining function¹¹, indicates that the presumptive testis-determining gene or small cluster of genes resides either on the short arm or on a pericentric region of the acrocentric human Y chromosome.

It is not likely that this presumptive testis-determining gene or set of genes specifies an enzyme or enzymes involved in sex steroid biosynthesis, because the initial biochemical pathways for synthesis of progesterone and oestrogen in the female, and of androgens in the male, are identical. Thus the same enzymes are used by ovary and testis (and to a certain extent by the adrenal) in the production of both male and female sex hormones. Moreover, development of the indifferent gonad into a testis under the influence of the Y chromosome begins as soon as the migration of primordial germ cells from the yolk sac to the gonadal ridge is complete, at which stage endocrine organs in general are not yet fully developed. Thus a cell surface protein or set of proteins concerned in cell-cell recognition and interaction might seem a likely candidate for the gene product or products responsible for initiating testicular differentiation (see below). Under the influence of the Y chromosome, somatic elements of the primitive gonadal ridge differentiate into interstitial cells and Sertoli cells, and as they do so primordial germ cells and Sertoli cells become enclosed within seminiferous tubules, whereas the interstitial cells remain outside. Interaction between the surfaces of somatic elements and germ cells seems essential for the organisation of testicular structure.

By serological methods, those proteins (or the sugar moieties of glycoproteins) which reside in the plasma membrane and are accessible to antibody can be recognised as the surface antigens of intact viable cells. We propose that one such antigen, H-Y (histocompatibility-Y), may be the direct product of the testis-determining gene postulated above.

Rejection of male skin grafts by females within an inbred strain of mice is due to H-Y antigen, found on the cells of all males, but absent from those of normal females (ref. 12; review in ref. 13). Using antibody raised in female mice against cells grafted from males of the same inbred strain, we have found that the cell surface component recognised as H-Y antigen has been highly conserved in vertebrate evolution^{14,15}. Our study of H-Y antigen expression in vertebrates has revealed that H-Y is not in fact specific for males, but rather for the heterogametic sex. Thus in birds and in certain species of Amphibia, female heterogamety of the ZZ/ZW type prevails, and in these animals it is the female whose cells express H-Y antigen¹⁵.

Widespread evolutionary conservation of a particular cell surface component indicates the invariant persistence of a specific function. Our hypothesis is that in the case of H-Y

antigen, this invariant specific function may be to direct the indifferent embryonic gonad to develop towards whichever mature gonad, testis or ovary, typifies the heterogametic sex of each species (testis in XY males and ovary in ZW females).

In mammals the proposed identity of H-Y antigen and the testis-determining gene product can best be tested on exceptional individuals whose gonadal sex does not coincide with their chromosomal sex. In mice and other rodents, a mutational suppression of the Y-linked testis-determining gene should give rise to ovaries in mutant XY individuals, since XO rodents are fertile females¹⁶. According to our hypothesis, such XY individuals would lack H-Y antigen. In man, a similar mutation should give 'streak gonads' in mutant XY subjects, because XO women are sterile¹⁷. Assuming that their streak gonads began as ovaries, these XY women should also type negative for H-Y antigen. On the other hand, a transfer of the testis-determining gene to an autosome, or alternatively, a mutational acquisition of testis-determining function by a dormant autosomal gene, should give rise to testes in mutant XX individuals. These we predict would be typed positive for H-Y antigen. Indeed our preliminary observations indicate that certain XX human males whose cells we have examined express H-Y antigen. Moreover XX mice that are 'sex-reversed' to males by the autosomal dominant, *Sxr* (ref. 18), are also positive for H-Y antigen. (D. Bennett, E. A. B., B. M. Cattenach, B. J. Mathieson, M. Scheid, and K. Yanagisawa, unpublished). The original breeding data and cytological observations indicate that if *Sxr* represents a piece of the Y translocated to an autosome, then that piece is so small as to be undetectable karyotypically¹⁸. Therefore the fact that *Sxr*/+ males express H-Y antigen clearly shows either that the testis-determining gene and the *H-Y* locus are extremely closely linked, or that the two are in fact one and the same.

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Expression of H-Y (male) antigen in phenotypically female *Tfm*/Y mice

THE X-linked testicular feminisation mutant (*Tfm*) in the mouse produces chromosomally male animals, X^{Tfm}/Y , with small testes but no other internal reproductive organs, and with female external genitalia¹. This condition results from

failure of target tissues to respond to testosterone, so that all characters of maleness that are testosterone-dependent are absent in males²⁻⁴.

In addition to the development of testes, there is one other attribute of maleness in the mouse which seems not to require testosterone; this is the expression of H-Y antigen, a cell surface component found on all cells of male mice, but on no cells of females⁵. Although some data are controversial, and while quantitative effects of testosterone can be envisaged, the bulk of evidence suggests that H-Y is expressed whenever the Y chromosome is present, and not otherwise.

Among the supporting evidence is the following: (1) Expression of H-Y antigen is not affected by the single-X compared with the two-X constitution; that is, XXY male mice have H-Y antigen whereas XO females do not⁶. (2) Testicular teratomas are H-Y⁺ when they have the Y chromosome but not when Y is lost as a consequence of aneuploidy that may occur on passage⁷. (3) Genetic data coupled with skin-grafting experiments suggest not only that H-Y is, determined by the Y chromosome, but that it may exist in different allelic forms in males of different strains⁸, this is, incidentally the best indication so far that the H-Y-determining gene of the Y chromosome is 'structural' rather than 'regulatory'. (4) Male haemopoietic cells colonising lethally irradiated females continue to express H-Y (ref. 9). (5) In spite of the evidence that neonatal castration may reduce the immunogenicity of male skin grafts¹⁰, deprivation of testosterone has not been shown to eliminate H-Y antigen qualitatively; nor did the administration of testosterone induce H-Y in females¹¹. (6) Evidence that newborn female skin that has been resident on males is rejected on regrafting to females, implying acquisition of H-Y in the male environment, is both claimed¹¹ and disputed¹². (7) The heterogametic sex, whether male or female, is H-Y⁺ in other vertebrate species¹³; in fact we must now recognise that the term 'male antigen' is a misnomer in reference to H-Y.

The most likely explanation for all these findings is that H-Y is controlled by a gene or genes on the Y chromosome, and that neither testosterone *per se* nor the male environment is required to elicit activity of this gene or genes.

The evidence is that all testosterone-dependent characters are lacking in *Tfm/Y* animals and that even the *Tfm/Y* embryo is unresponsive to testosterone⁴. Therefore the finding that we report here, namely that *Tfm/Y* mice are H-Y⁺, is strong evidence that the expression of H-Y is testosterone independent.

Mice from the Harwell *Tfm* stock are maintained by crossing *Tfm*+/+/+ *Bloto*+/+ *Ta*+/+ *Y* and *Tfm*+/+/+ *Ta*+/+ to +/+/+ *Blot*/Y in alternate generations. For the tests described below, the *Tfm* animals were *Tfm/Y* from this stock, the control females were their *Tfm*+/+/+ *Ta*+/+ siblings, and the control males were +/+/+ *Blot*/Y siblings.

Anti-H-Y sera were prepared in C57Bl/6 (abbr B6) females by repeated grafting of B6 male skin. The anti-H-Y activity of such sera is demonstrable by the complement-dependent cytotoxicity assay on sperm¹⁴ or dissociated epidermal cells¹⁵. We used 3 types of experiment: (1) The cytotoxicity assay with anti-H-Y serum on epidermal cells from *Tfm/Y* mice (Fig. 1).

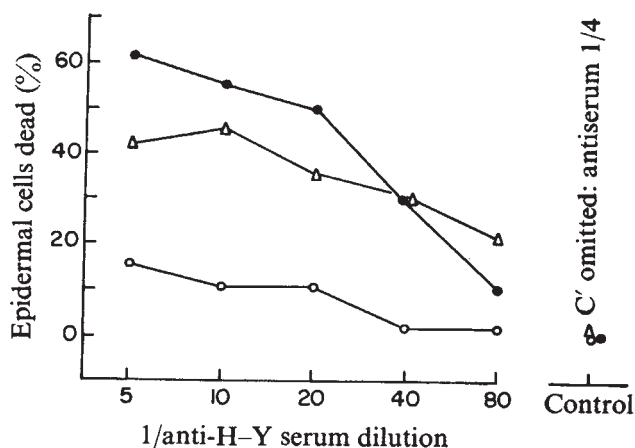


Fig. 1 Demonstration of H-Y antigen on dissociated epidermal cells of *Tfm/Y* mice by the complement-dependent cytotoxicity assay test with anti-H-Y serum. The data in this figure are from a representative test with two mice of each genotype. The values shown are mean readings for the pairs of mice. The anti-H-Y serum was preabsorbed twice with female epidermal cells (4 parts serum undiluted: 1 part packed cells) at 0-4 °C. Complement source was selected rabbit serum, absorbed with thymocytes, lymphocytes, and epidermal cells in the presence of EDTA (see ref. 15), diluted 1/6 before use. Percentage dead epidermal cells (ordinate) calculated by the formula $\{(a-b)/(100-b)\} \times 100$ where *a* is the % dead cell count (Trypan blue stained cells) after incubation with antibody and complement; and *b* is the similar count with antiserum omitted. This somewhat lower reaction of *Tfm/Y* epidermal cells, compared with control male cells is not significant, and was not a feature of other similar tests. Δ, *Tfm/Y*; ●, +/+/+ *Blot*/Y (normal male control); ○, *Tfm*+/+/+ *Ta*+/+ (normal female control).

(2) Absorption of anti-H-Y serum with spleen cells from *Tfm/Y* mice followed by testing for cytotoxicity on sperm (Fig. 2). (3) Grafting of *Tfm/Y* skin to B6 females to determine whether this would presensitise them for a second-set response to subsequent grafting of B6 male skin (Table 1). Figure 1 gives one representative example out of a total of five positive cytotoxicity tests with anti-H-Y serum on *Tfm/Y* epidermal cells. Figure 2 shows two positive absorption tests with *Tfm/Y* spleen cells. Table 1 summarises the results of the grafting experiments, in which *Tfm/Y* skin grafts sensitised B6 females to subsequent B6 male skin grafts. In all of these tests, cells from *Tfm/Y* mice have proved indistinguishable from those of normal males in their expression of H-Y.

We conclude that H-Y is not testosterone dependent. Since the *Tfm/Y* animals have small testes, which seem fairly normal at foetal to neonatal stages⁴, one must consider whether it is the presence of testes which determines the production of H-Y antigen or whether the presence of the Y chromosome itself is sufficient. The concept that some influence of a gonad could affect surface antigenicity of cells of other tissues, seems very difficult to accommodate within existing knowledge. Furthermore, it is not yet certain when Y-antigen expression begins during development, and so it may in fact precede

Table 1 Second-set rejection of B6 male skin by B6 females previously grafted with *Tfm/Y* skin*

First graft (number of B6 females grafted)	Rejection time (d) of B6 male skin grafted 2-3 months later	MST† (confidence limits)
<i>Tfm/Y</i> (15)	11, 12, 13, 13, 13, 14, 14, 14, 15, 15, 16, 16, 17, 17.	14 (12.7-15.4)
+ + <i>Blot</i> /Y; male controls (13)	12, 12, 15, 15, 16, 16, 16, 17, 17, 18, 18, 22, 22.	15.5 (13.5-17.8)
<i>Tfm</i> +/+/+ <i>Ta</i> +/+; female controls (8)	12, 16, 17, 18, 19, 21, 21, 40.	18 (16.4-19.7)

*Summary of data for two experiments involving four *Tfm/Y* graft donors and three donors for each of the two control groups; these donors also provided spleen cells and tail epidermal cells for the serological tests (Figs 1 and 2). Trunk skin grafted according to Billingham and Medawar¹⁷, using Band-Aids instead of plaster casts; rejection times scored visually.

†Median survival time (MST) and confidence limits of the median calculated by the method of Litchfield¹⁸.

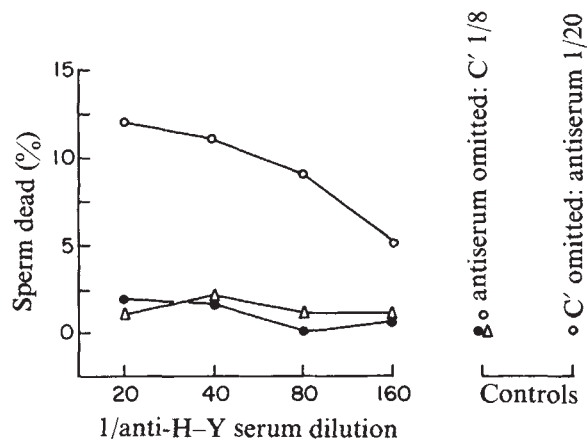


Fig. 2 Demonstration of H-Y antigen on *Tfm/Y* spleen cells by absorption of cytotoxic activity from anti-H-Y serum. The data shown illustrate a representative test. Each point represents the mean value obtained when spleen cells from two different mice of the same genotype were tested separately. The anti H-Y serum (preabsorbed with female epidermal cells, see footnote to Fig. 1) was diluted 1/4 and absorbed in 0.1-ml volumes with spleen cells from *Tfm/Y* mice or control mice as indicated, for 30 min at 0–4 °C; after centrifugation to remove the cells, the absorbed serum was titrated against sperm for residual cytotoxic activity, using horse serum diluted 1/8 as the complement source. The tests were read 'blind' with absorbed antiserum sample coded. Percentage sperm dead (ordinate) was calculated by the formula $\{(a-b)-(100-b)\} \times 100$, where a is number of sperm dead, and b is the % dead sperm in original suspension (method described in detail elsewhere¹⁴). Sperm were obtained from unrelated normal mice. H-Y antiserum absorbed with: Δ , *Tfm/Y*; $\bullet$, $+/+Blo/Y$ (normal male controls); $\circ$, *Tfm* $+/+Ta+$ (normal female controls).

testis formation. We prefer to consider that the detection of H-Y in these *Tfm/Y* phenotypic females with a Y chromosome constitutes further compelling evidence that H-Y is indeed Y dependent, not male dependent.

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Effect of morphine on a depolarising dopamine response

THE structural complexity of nervous tissues and the presence of non-neuronal elements have hindered investigations into the actions of morphine at the cellular level in the vertebrate central nervous system, and contribute to the difficulty of determining whether morphine acts pre- or postsynaptically. Although presynaptic effects of morphine are documented, to our knowledge there has been no demonstration of an effect

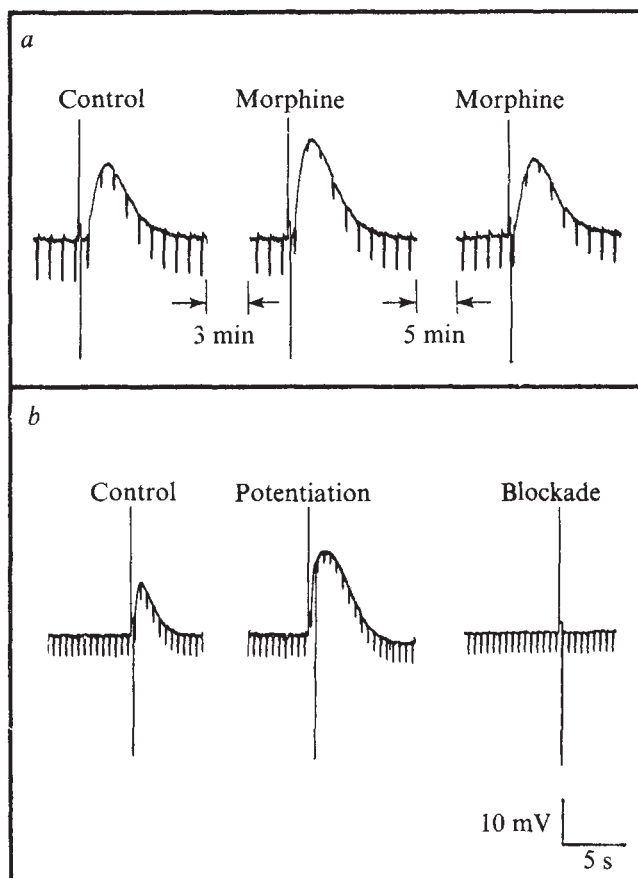


Fig. 1 *a*, Potentiation of the DA response by low morphine concentrations. DA iontophoretic charge, 500 nC. Current pulse amplitude was maintained constant at 0.26 nA for the duration of the experiment. *b*, Potentiation of the DA response by low morphine concentrations followed by blockade of the response at higher morphine concentrations in the same cell. Morphine added to the bath (0.5 μ M) caused an increase in the DA response amplitude. An excess of morphine (24 μ M) blocked the DA response. Potentiation could be attained with 0.2–0.6 μ M morphine and blockade with 8–12 μ M morphine. DA iontophoretic charge, 300 nC. Current pulse amplitude was maintained constant at 0.15 nA for the duration of the experiment. Morphine in the concentration range tested (0.1–50 μ M) had no effect on the passive membrane properties. Polarising current pulses were passed across the membrane to determine conductance changes. Standard electrophysiological techniques using a bridge circuit recorded membrane potential and passed polarising current intracellularly simultaneously. Glass microelectrodes filled with either potassium acetate or chloride and with resistances greater than 30 M Ω were used for recording. During recording, the 35-mm Falcon culture dish on which the cells were grown remained covered except for a 1-cm hole in the centre to allow for entry of electrodes. The culture dish was maintained at 37 °C and gassed continually with a stream of water-saturated 10%–95% carbon dioxide–air mixture. There was negligible evaporation of growth medium during any one recording session. 3,4-Dihydroxyphenylethylamine (dopamine)-HCl (Sigma) was prepared in aqueous 1 M concentrations (pH 3–4) and used to fill iontophoretic glass microelectrodes, the final resistance of which was adjusted to 10–20 M Ω by bumping the electrode tip.

of morphine on postsynaptic neurotransmitter receptors using intracellular recording techniques.

We have studied the effects of morphine on the dopamine response of the somatic cell hybrid 108CC-15. The cell line is a product of the fusion of cells from the mouse neuroblastoma N18TG2 and the rat glioma C6-BU-1 and expresses a number of neuronal phenotypes¹⁻⁴. It is a homogeneous population of cells, allowing intracellular recording from single cells free of organisational complexity or synaptic input. The biochemical and electrical properties of the cell line have been described in detail elsewhere¹.

When dopamine (DA) is applied iontophoretically, a depolarising response, mediated by a conductance increase, is seen in 64% of the cells. The average resting membrane potential was -42.5 mV with an average input resistance of 39 m Ω . The DA response has a reversal potential of -15.9 mV (± 1.2). Repeated application of DA at close time intervals resulted in desensitisation of the response. The response is blocked by the catecholamine antagonists bulbo-capnine, chlorpromazine, and phentolamine; the dopamine metabolite 3-methoxytyramine, and the acetylcholine antagonists hexamethonium and α -bungarotoxin, are without effect. Details of cell growth for this electrophysiological study and additional characteristics of the DA response are published elsewhere²².

Fresh solutions of morphine sulphate (Mallinckrodt) and naloxone HCl (Endo Laboratories) were prepared before each

experiment. After each drug application to the bath, adequate time was allowed (5–10 min) to allow equilibration of the drug in the medium. Morphine was applied iontophoretically from glass micropipettes filled with 1% morphine sulphate. Morphine (0.1 – 50 μ M) and naloxone (1 – 10 μ M) had no effect on the passive membrane properties. Current pulses were injected continuously during drug applications and demonstrated that there were no changes in membrane resistance or membrane potential.

Morphine at low bath concentrations (0.2 – 0.6 μ M) increased the DA response amplitude up to 75% above control levels (Fig. 1a). The increase was transient, since the response amplitude was observed to return to control levels and in most cases, it was not possible to repeat the potentiation on the same cell after the initial exposure to a low morphine concentration. The potentiation was also observed with iontophoretic application of morphine.

Higher bath concentrations of morphine (8 – 12 μ M) blocked the DA response and it remained blocked as long as morphine was present in the bath. Figure 1b shows potentiation of the DA response in a single cell at low concentrations but that on addition of more morphine to the bath, the DA response was blocked. Iontophoretic application of morphine blocked the DA response and this block was dose dependent and reversible after diffusion of the drug into the medium (Fig. 2a). When morphine was added to the bath in sufficient concentration to block the DA response, addition of equimolar naloxone reversed the blockade and the DA response recovered to control levels (Fig. 2b). This observation agrees well with the known antagonist action of naloxone on morphine.

In both peripheral and central nervous tissues, various lines of evidence have suggested both a pre- and postsynaptic effect of morphine. A presynaptic action of morphine is argued from studies of the uptake, metabolism and release of neurotransmitters⁵⁻¹³. Inferences on the postsynaptic action of morphine are based on morphine interaction with neurotransmitter antagonists¹⁴⁻¹⁷. In a separate study, Bradley and Dray¹⁸ found both potentiation and inhibition of neurones when morphine was applied iontophoretically to brain stem. The potentiation was observed to "desensitise". Our results support the view that morphine acts at least postsynaptically.

Pert and Snyder¹⁹ first demonstrated that opiate receptors exist in the brain; Klee and Nirenberg²⁰ have demonstrated that 108CC-15 has opiate receptors with characteristics similar to brain receptors. That morphine influences the DA response suggests the existence of some relationship between opiate receptor binding and neurotransmitter receptors. Although Pert and Snyder²¹ have shown that catecholamines do not block binding of morphine to its receptor, their results do not preclude a specific interaction of morphine with the DA receptor.

We thank Dr Berndt Hamprecht on whose cell line this work was done and Drs David Carpenter and James Blosser for their discussions and criticisms.

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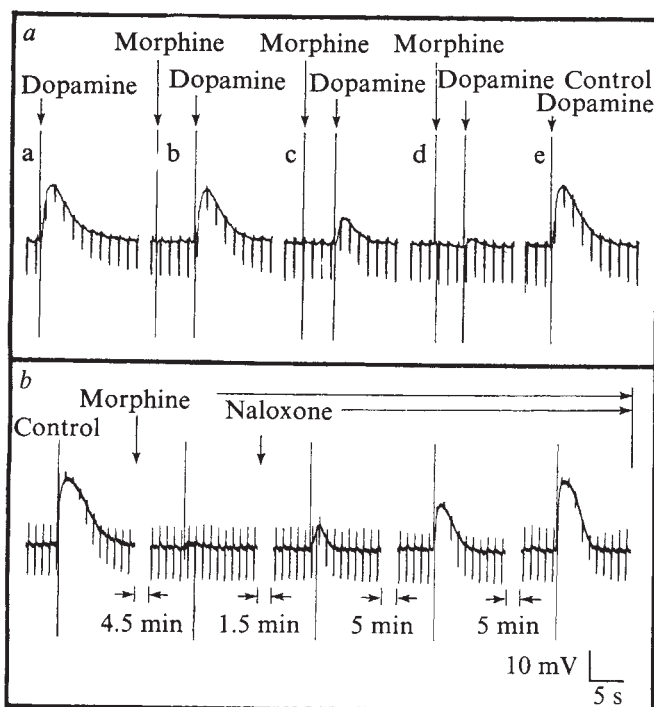


Fig. 2 a, Dose-response characteristic of the morphine blockade. Morphine applied iontophoretically in increasing quantities decreased the DA response amplitude in a dose-dependent fashion. The blockade was reversible after diffusion of the drug into the medium and the blockade could be repeated on the same cell. In recordings b, c, and d the first stimulus represents morphine application and the second represents DA application. The time between drug applications was 2 min. a, 300 nC DA; b, 50 nC morphine, 300 nC DA; c, 100 nC morphine, 300 nC DA; d, 200 nC morphine, 300 nC DA; e, 300 nC DA. Current pulse amplitude was maintained constant at 0.5 nA for the duration of the experiment. b, Reversal of the morphine block by naloxone. After sufficient morphine was added to the bath to block the DA response (7.3 μ M), an equimolar amount of naloxone was added and the DA response returned to control levels. DA iontophoretic charge, 300 nC. Current pulse amplitude was maintained constant at 0.3 nA for the duration of the experiment. Naloxone added to the bath alone had no effect on the membrane passive properties nor the DA response. In similar experiments, longer time periods were allowed after the addition of morphine to the bath to ensure equilibration and that return of the DA response after addition of naloxone to the bath was not purely coincidental.

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Receptor potential and impulse initiation in two varieties of reptilian muscle spindle

AMONG muscle spindles, those in snake and lizard are the simplest in structure, consisting of a single intrafusal muscle fibre innervated by a single sensory and several motor nerve fibres. Two varieties of spindle occur: one type, the long-capsule spindle has a longer and more slender capsule than the other, the short-capsule spindle¹. They differ in their responses to stretch; the response of the long-capsule spindle depends mainly on degree of stretch while the short-capsule spindle responds both to velocity and amplitude of stretch^{2–4}.

Sensory transduction in muscle spindles involves the following steps: transmission of mechanical stimulus (stretch) through intrafusal fibres, generation of receptor potential in the sensory terminals and impulse initiation in the afferent fibre. While there is information on the mechanical transmission in reptilian spindles⁵ and on the characteristics of the impulse initiating process⁶, there has been no study of the receptor potential in long- and short-capsule reptilian spindles in response to stretch. We report here some features of receptor potential characteristic of each type of snake spindle which may account for differences in the early adaptation of impulse discharge between the two types of spindle.

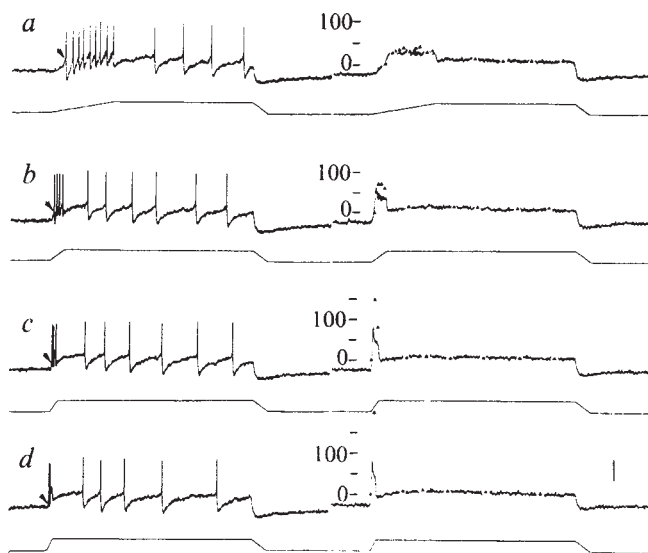
Muscle spindles in ventral costocutaneous muscles of garter snake (*Thamnophis*) were used. The technique for dissection of the muscle and the ionic composition of the bathing solution have been described elsewhere⁷. After visual identification of the type of spindle under the dissecting microscope and dark field illumination, a single spindle was isolated with some extrafusal fibres which served as a support. Each end of the muscle was tied to a nylon rod which was connected to one of a pair of Brush pen motors. The muscle spindle was stretched to both sides by a computer (LINK) controlled activation of Brush pen motors at a rate of once every 5 s. The isolated spindle was brought near the interface between snake Ringer's solution and liquid paraffin and the nerve was lifted into liquid paraffin on one of a pair of glass pipettes filled with agar-Ringer. The other pipette was placed in the Ringer's solution. Each pipette was connected to one of a pair of half calomel cells and signals from the nerve were amplified by a PAR amplifier. Most of the experiments were done at the muscle length at which slack was just taken up. Tetrodotoxin (TTX) of 10^{-7} – $10^{-6}\%$ was used to block impulse initiation in the ending. After each experiment the nerve was crushed with a pair of fine forceps at the region close to the capsule. In all cases examined no noticeable movement artefact was observed. Experiments were performed at room temperature (about 25 °C).

Figures 1 and 2 illustrate examples of responses of a short-capsule and a long-capsule spindle, respectively, to ramp-and-hold stretch of increasing velocity (from a to d) before (left column) and after (right column) application of TTX. Several components may be recognised in the dynamic component of the receptor potential particularly in short-capsule spindles. At the beginning of stretch there is a steep rise of receptor potential (initial component) and at the end of the ramp the potential swings back to a lower level (dynamic-static fall)

often making an undershoot (dynamic-static undershoot). Between the initial component and the dynamic-static fall the potential shows either a plateau or gradual decay or increase depending upon the type of spindle and the velocity and amplitude of stretch. During the hold phase of stretch the potential gradually declines (adaptive static fall) and at the end of stretch the potential may show "release undershoot" before returning to the original level (the above terminology adopted from ref. 8). In the long-capsule spindle the initial component and the dynamic-static undershoot are both usually lacking. The initial component may correspond to the initial burst of discharge which often occurs in short-capsule, quite rarely in long-capsule spindles. The dynamic-static undershoot may correspond to the pause of impulse activity which occurs just after the ramp as may be seen in Fig. 1. The above features of receptor potentials characteristic of the short-capsule and the long-capsule spindles are strikingly similar to those recorded from the primary and the secondary endings, respectively, in cat muscle spindles⁸. In order to examine the relation between dynamic components of receptor potential and impulse discharge the threshold depolarisation of receptor potential (zero frequency on ordinate) for initiating a train of impulses was determined by extrapolating the approximately linear relation between mean static frequency and receptor potential 0.5 s after the ramp phase of stretch of varying amplitude. The scaling of frequency on the ordinate was then adjusted so that the mean static frequency and the receptor potential 0.5 s after the ramp coincide each other. The results thus obtained are shown in Figs 1 and 2 as filled triangles superimposed on each receptor potential. In both types of spindle when the velocity of stretch is slow the frequency of impulse discharge follows relatively well the dynamic component of receptor potential. As the velocity of stretch increases the discrepancy between the two parameters increases progressively. Particularly in short-capsule spindles the dynamic impulse frequency rises far above the level of receptor potential.

The progressive discrepancy between discharge frequency and the receptor potential with the increase in velocity of stretch could be accounted for by accommodative process occurring

Fig. 1 Responses of a short-capsule spindle to ramp-and-hold stretch of increasing velocity (from a to d, see each lower trace) before (left column) and after (right column) application of 10^{-6} TTX. Records were all reproduced from tape. A pair of records in a single row are responses to an identical stretch. Velocity of stretch (mm s^{-1}): a, 1.0; b, 5.0; c, 10.0; d, 15.0. Amplitude of stretch, 500 μm . Arrows indicate threshold level of receptor potential for the first spike. Triangles, frequency of impulse discharge before application of TTX (explanation of scaling in text). Ordinate, impulses s^{-1} . Time calibration, 0.5 s. Voltage calibration (250 μV) applies to all records of response.



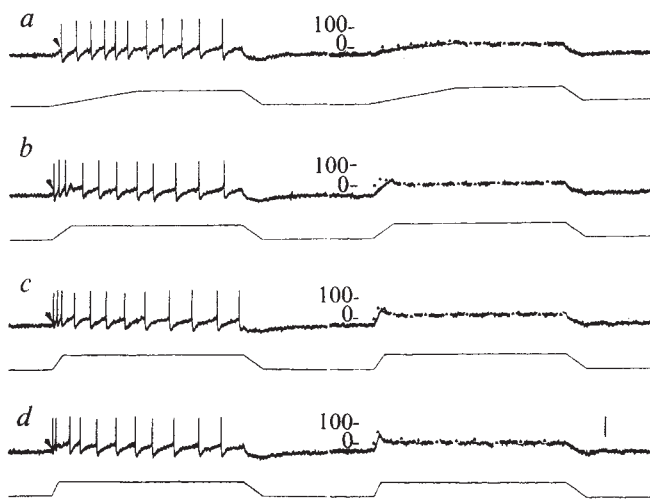


Fig. 2 Responses of a long-capsule spindle to ramp-and-hold stretch of increasing velocity (from *a* to *d*) before (left column) and after (right column) application of 10^{-6} TTX. Records are arranged in the same way as in Fig. 1. Velocity of stretch (mm s^{-1}); *a*, 1.0; *b*, 5.0; *c*, 10.0; *d*, 15.0. Amplitude of stretch, 350 μm . Arrows and triangles, threshold depolarisation for the first spike and impulse frequency plotted as in Fig. 1 respectively. Ordinate, impulses s^{-1} . Time calibration, 0.5 s. Voltage calibration (200 μV) applies to all records of response.

in the spike initiating site. In fact, with decrease in stretch velocity substantial increase in the threshold depolarisation for the first spike may be seen in Figs 1 and 2 (arrows). On the basis of accommodation it may be predicted that the larger the dynamic component of receptor potential the larger discrepancy would occur between impulse frequency and receptor potential as illustrated in Figs 1 and 2. Another possibility for the discrepancy may be the preferential suppression of the dynamic component of receptor potential by TTX. This seems less probable because (1) after application of TTX the dynamic discharge is always the last to be blocked, (2) higher concentration of TTX (10^{-5}) does not seem to affect appreciably the receptor potential and (3) as in Figs 1 and 2 the dynamic impulse frequency and the dynamic receptor potential at low velocity of stretch coincide rather well with each other. Whatever the underlying mechanism is, however, it may be concluded that the basis for the difference in stretch-velocity sensitivity between the two types of spindle is due mainly to the difference in the dynamic component of the receptor potential. Taking the previous findings^{5,6} into consideration it follows that either the mechanical event occurring at the ultrastructural level particularly in the sensory ending-intrafusal fibre contact region and/or in the ionic mechanisms for generating receptor potentials in the sensory ending may be responsible for the stretch-velocity sensitivity of snake muscle spindles.

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Binding of aldosterone by mitochondria-rich cells of the toad urinary bladder

ALDOSTERONE causes a large increase in the active transport of sodium by the urinary bladder¹ of the toad. This response, analogous to the effects of the steroid on the kidney, has been the object of considerable biochemical study². Aldosterone also enhances the stimulation by neurohypophyseal hormones of sodium and hydro-osmotic flux^{3,4}. Because the response in sodium transport seems quantitatively related to the increment of cyclic AMP generated by vasopressin⁵, augmentation of the sodium response is presumably related to the larger increment of intracellular nucleotide induced by a given dose of vasopressin in steroid-treated bladders⁶. Using a technique for separating the two major cell types of the bladder mucosal epithelium—the granular (G) and the mitochondria-rich (MR) cells—we found that a response to neurohypophyseal hormones, as reflected by hormone-induced increases in the intracellular cyclic AMP content, is apparently limited to the MR cell⁷. This apparent synergism of the neurohypophyseal and steroid hormones on transport suggests that their loci of action are in close proximity. We therefore examined the binding of ³H-aldosterone by the two major cell types.

To measure the specific binding of ³H-aldosterone by mineralocorticoid receptors, we incubated paired sets of 6–10 intact hemibladders in separate baths. One bath (³H-aldo) contained ³H-aldosterone alone in various concentrations, while the paired bath (control) contained the same concentrations of ³H-aldosterone plus a 1,000-fold excess of unlabelled steroid. After incubation for 90 min, the mucosal cells were removed by immersing the bladders in EDTA–Ringer solution. The MR and G cells of each set of bladders were separated by density gradient centrifugation⁷ and the ammonium-sulphate precipitable ³H-aldosterone was measured in each of the four preparations. The difference in the ³H-aldo and control values for each cell type was assumed to represent specifically-bound or displaceable steroid (Table 1).

We found that the amount of displaceable ³H-aldosterone in MR cell preparations was related directly to the concentration of labelled steroid in the bath. With the exception of a single experiment, there was no significant binding of displaceable aldosterone by G-cell preparations. We also measured the specific binding of corticosterone, the natural glucocorticoid of *Bufo marinus*, by the two cell types. ³H-corticosterone (New England Nuclear, 84 Ci mmol^{-1}), 10^{-8} M, and a 1,000-fold excess of unlabelled corticosterone, were used as in the aldosterone binding studies. The amount of displaceable corticosterone bound by the G cells (2.6×10^{-14} mol per mg protein) was equivalent to that bound by the MR cells (2.95).

The displaceable aldosterone bound by the MR cells was considerably greater in April than values measured during September to March. This increased level of binding persisted until late August when successive experiments at the level of 5×10^{-9} M ³H-aldosterone showed a progressive decrease in the amount of steroid bound by the MR cell (from the expected 28.0) to 9.5, 8.9, 2.9 and 2.1×10^{-14} mol ³H-aldosterone per mg protein. This compared with a value of 2.15 obtained the previous March (Table 1). This marked variability in the amount of aldosterone bound by the MR cells corresponds and may be related to the well known seasonal variations in the responsiveness of the toad bladder transport mechanisms to mineralocorticoid hormones.

The mineralocorticoid/glucocorticoid selectivity of the receptor binding aldosterone was examined by measuring the efficacy with which deoxycorticosterone (DOCA) and cortisol displaced ³H-aldosterone. Hemibladders were incubated in three sets. In addition to ³H-aldo and control sets used in previous experiments, the third set was exposed to either DOCA or cortisol in 200-fold excess as well as ³H-aldosterone.

Table 1 Specifically bound ^3H -aldosterone in separated mitochondria-rich and granular cells

	^3H -aldosterone ($\text{M} \times 10^{-9}$)	Mitochondria-rich cells			Granular cells		
		^3H -aldo	Control	Net	^3H -aldo	Control	Net
November–March	1.2	1.38	0.84	0.54	0.73	0.74	0
	2.0	1.32	0.12	1.20	0.16	0.12	0.04
	2.2	2.59	1.79	0.80	1.23	0.94	0.29
	2.8	3.09	1.68	1.41	0.65	0.48	0.17
	5.0	3.05	0.89	2.15	1.18	0.87	0.30
April–June	20.0	8.50	4.16	4.34	4.15	5.57	0
	1.0	8.70	0.07	8.6	1.50	1.86	0
	4.0	24.00	0	24.0	4.70	6.50	0
	10.0	68.80	0	68.0	18.20	1.68	16.5
	40.0	88.00	1.80	86.2	1.77	1.91	0

Toads (*B. marinus*) of Colombian origin (Tarpon Springs Inc.) were kept in 0.6% saline for at least 3 d to suppress endogenous aldosterone. Cells were derived from two sets of 8–12 urinary hemibladders incubated for 90 min in Ringer containing NaCl, 85 mM; KCl, 4 mM; NaHCO_3 , 17.5 mM; KH_2PO_4 , 0.8 mM; MgSO_4 , 0.8 mM; CaCl_2 , 1.5 mM; dextrose, 10 mM; and ^3H -aldosterone (New England Nuclear, 91.6 Ci mmol $^{-1}$), in the indicated concentrations. The control bath also contained a 1,000-fold excess of unlabelled aldosterone. The mucosal cells were removed by incubation in EDTA–Ringer and the MR and G cells separated by centrifugation on a discontinuous density gradient of Ficoll $^{\text{®}}$. The MR and G cells from the ^3H -aldo and control tissues were sonicated and the radioactivity measured in the ammonium sulphate (50% saturated) precipitable fraction of the 110,000g supernatant. The specifically bound steroid in each experiment, the difference between the ^3H -aldo and control, is expressed as $\text{mol} \times 10^{-14}$ per mg protein.

As Table 2 shows, DOCA, a potent mineralocorticoid, completely displaced ^3H -aldosterone from the MR cells. Cortisol, primarily a glucocorticoid, displaced only about 24% of the specifically-bound aldosterone, indicating high mineralocorticoid specificity for the ^3H -aldosterone binding sites.

The subcellular distribution of displaceable ^3H -aldosterone was determined in unseparated mucosal cells. Intact hemibladders were incubated as 10 paired sets of ^3H -aldo and control tissues. Both bathing solutions contained 10^{-8} M ^3H -aldosterone and the control bath also contained 10^{-8} M unlabelled aldosterone. After incubation for 90 min the mucosal cells were removed and the two suspensions of mucosal cells were disrupted by pressure homogenisation, which leaves the nuclei intact. The homogenates were fractionated by differential centrifugation and ammonium sulphate precipitation. In bladders incubated in ^3H -aldosterone alone, 1,222 c.p.m. representing approximately 12% of the steroid in the homogenate, sedimented with the nuclei (Table 3). Receptor-dependent uptake of aldosterone by the nucleus is demonstrated by the large reduction in the amount of labelled steroid sedimenting with the nuclei prepared from control tissues (4.6%). The ^3H -aldosterone bound by the ammonium sulphate precipitate prepared from the supernatant fraction of the cell also demonstrated significant displacement by unlabelled steroid. These data, which confirm earlier studies 8 of subcellular localisation of aldosterone, indicate that the steroid is bound by specific receptors in both the nucleus and the cytoplasm of the mucosal cell.

The identification of the MR cell as the aldosterone-binding locus in the bladder mucosal epithelium is reinforced when

displaceable steroid is determined in the nuclei from isolated MR cells. Using incubation procedures described earlier, the MR cell nuclei from ^3H -aldo (10^{-8} M) and control tissues were isolated. The nuclei from the ^3H -aldo set contained 7,650 c.p.m., which when compared with only 60 c.p.m. in the control nuclei, indicates nuclear accumulation of aldosterone by a specific receptor mechanism in the MR cells.

Our data demonstrate the specific, concentration-dependent binding of aldosterone by the MR cells and localisation of the steroid within the nucleus. These results, together with our demonstration that aldosterone induces the synthesis of protein in MR cells 10 , imply a transcriptional effect of aldosterone.

Table 3 Subcellular distribution of ^3H -aldosterone in isolated mucosal cells

Subcellular fraction	^3H -aldo	Control
Nucleus	12.0%	4.6%
Postnuclear pellet	10.6	11.8
Supernatant	84.1	87.5
$-(\text{NH}_4)_2\text{SO}_4$ precipitate	8.4	5.1

Paired sets of hemibladders were incubated as in Table 1; the concentration of ^3H -aldosterone was 10^{-8} M. After 1.5 h mucosal cells were removed by incubation in Ringer containing EDTA (2 mM) with no Ca^{2+} or aldosterone present. Mucosal cells were disrupted by pressure homogenisation; the samples were equilibrated for 30 min with nitrogen at 1,350 pounds inch $^{-2}$ before release 9 . Nuclei were sedimented by centrifugation at 600g for 20 min. The 600g supernatant was centrifuged at 110,000g for 30 min (postnuclear pellet) and protein in this supernatant precipitated by ammonium sulphate (50% saturated). Radioactivity was measured by liquid scintillation and the radioactivity in each fraction expressed as the percentage of that in the original homogenate.

Table 2 Effects of DOCA and cortisol on aldosterone binding in toad bladder MR cells

Date	³ H-aldo	Control	Competing steroid		%Displacement of ³ H-aldo by competitor
			DOCA	Cortisol	
8/28	15.7	6.2	5	—	> 100
8/30	14.7	5.8	—	12.3	27
9/10	7.4	4.5	—	6.8	21
9/18	3.6	1.4	1.2	—	> 100

Three sets of eight hemibladders were used: ^3H -aldo, hemibladders incubated in 5×10^{-9} M ^3H -aldosterone; control, hemibladders incubated in 5×10^{-9} M ^3H -aldosterone plus 5×10^{-6} M unlabelled aldosterone; and either DOCA or cortisol, hemibladders incubated in 5×10^{-9} M ^3H -aldosterone plus the competing steroid (10^{-6} M). The values given (in $\text{mol} \times 10^{-14}$ ^3H -aldosterone per mg ammonium sulphate-precipitable protein) are the amounts of ^3H -aldosterone bound by the MR cells in each set of hemibladders.

These observations, together with earlier evidence that the MR cell is the target of neurohypophyseal hormones 7 , indicate that the MR cell is the initial locus of action of natriuretic hormones. Other investigators have suggested that the MR cell is the site of action of aldosterone 11 . Although the G cells may well participate in the hormone-induced sodium and hydro-osmotic fluxes 12 , our data indicate a unique role for the MR cells in the hormonal regulation of transport in this tissue. Indeed, the unique stellate arrangement of G cells around the less numerous MR cells 13 raises the possibility that the two cell types may act cooperatively in the tissue's response to peptide and steroid hormones.

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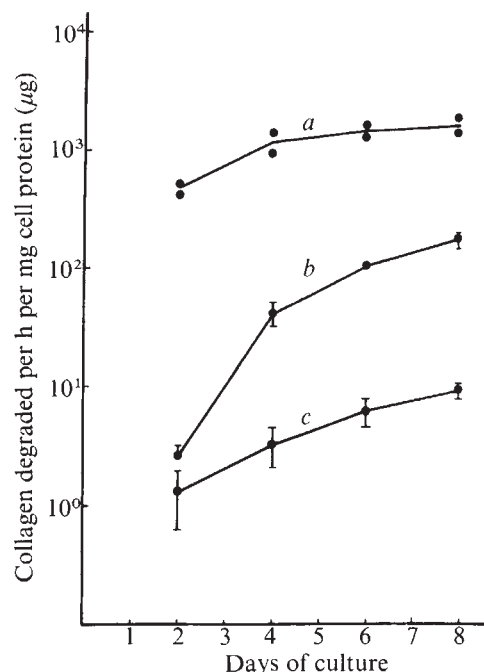


Fig. 1 Stimulation by CB of collagenase in cultures of rabbit synovial fibroblasts. CB was made up in a stock solution in DMSO (5 mg ml⁻¹) and diluted in DMEM before use. DMSO (0.1-1%) had no effect on collagenase production or activity. Medium containing 10% FCS was replaced with serum-free medium at time 0 and subsequently collected every 48 h. Collagenase activity was assayed using ¹⁴C-labelled collagen fibrils as substrate (reaction mixture: 100 µl 0.2% collagen, 80 µl culture medium, 20 µl 0.01 M *p*-chloromercuribenzoate and 100 µl 0.1 M Tris-HCl, pH 7.6, 0.03 M CaCl₂). Incubation was for 18 h at 37°C. The protein content, determined by the method of Lowry *et al.*¹⁴, of cells adhering to dishes at the end of the experiment did not differ significantly among groups. Duplicate assays were performed on all samples. Values for CB at 10 µg ml⁻¹ represent two dishes (a). Eight dishes received CB at 1.0 µg ml⁻¹ (b) and there were four control dishes (c); means ± s.e.m. are shown for control samples, and those treated with CB at 1.0 µg ml⁻¹. The cumulative enzyme release from the cells treated with CB at 1.0 µg ml⁻¹ was different from the controls (*P* < 0.01).

Cytochalasin B increases collagenase production by cells *in vitro*

COLLAGENASE is one of the major enzymes involved in the remodelling of connective tissues in normal and pathological states. Specific collagenases have been isolated from the culture media of many tissues *in vitro*¹ and have been found in culture media from various cells in monolayer culture, including rabbit synovial fibroblasts², guinea pig macrophages^{3,4} and human skin fibroblasts⁵. For a better understanding of the mechanisms by which collagenases are synthesised and released from cells, studies have focused on agents that stimulate these processes. Endotoxin³ and lymphokines⁴ induce release of collagenase from guinea pig macrophages, and a sustained release of collagenase and neutral protease from rabbit synovial fibroblasts was produced by phagocytosis of polystyrene latex particles⁶. It seems likely that all these agents affect cell membrane function, and so we have tested the effect on collagenase production by rabbit synovial fibroblasts of cytochalasin B (CB), which perturbs membranes in ways similar to phagocytosis⁷.

Fibroblast-like cells grown out from primary explants of rabbit synovium were dissociated mechanically or by a trypsin-EDTA mixture and then grown to confluence in glass prescription bottles in Dulbecco's modified Eagle's medium (DMEM) fortified with 10% foetal calf serum (FCS). Cells from the sixth to fourteenth passage generations were used. Human cells derived from synovial membrane were obtained in similar fashion from tissue provided by Mr Alan Murley, Orthopaedic Section, Addenbrooke's Hospital, Cambridge. Human skin fibroblasts grown from two normal children and from a child with the Hurler syndrome (type I mucopolysaccharide storage disease) were the gift of Dr William Sly, Department of Paediatrics, Washington University, St Louis, Missouri.

For studies with CB, cells were passaged into Nunclon plastic cell culture dishes (diameter 25 mm) and used when they approached confluence. As Figure 1 shows there was a large increase in collagenase activity in culture medium from CB-treated rabbit cells (1 µg ml⁻¹). CB caused cells to retract cytoplasm around nuclei, leaving thin cell processes extending in multiple directions, and this effect was greater in cells exposed to 10 µg ml⁻¹; at 0.1 µg ml⁻¹, CB caused

no morphological changes and no increase in collagenase activity. Concentrations of CB > 10 µg ml⁻¹ in the absence of FCS were toxic to cells; many became detached from the dishes. It is not essential to preincubate cells with CB in the presence of FCS to stimulate collagenase release, but removal of CB from medium resulted in a prompt fall in collagenase release to control levels.

Studies with the human fibroblasts have given essentially similar results (Table 1). Dissociated cells were added to duplicate dishes in dilutions of zero (2 × 10⁵ cells ml⁻¹), 1:1 (10⁵ cells ml⁻¹) and 1:3 (0.5 × 10⁴ cells ml⁻¹). CB (1.0 µg ml⁻¹) was then added only to the cells diluted 1:1. More collagenase was released from untreated Hurler cells than from untreated normal cells, as noted by others (unpublished results of Z. W., W. Sly and J. T. Dingle). The fractional increase over control levels of enzyme release induced by CB was, however, greater in the fibroblasts derived from normal skin.

The cumulative collagenase activity released from human fibroblast-like cells (eighth passage) derived from synovium removed during insertion of a prosthetic hip in a 75-yr-old woman with degenerative arthritis was determined, using a protocol similar to that described in the legend of Fig. 1. In the 6 d of culture after addition of serum-free medium containing CB (1.0 µg ml⁻¹) there was a cumulative mean collagenase activity (U) in four dishes of 1,423 ± 78 (s.e.m.) compared with the lower level of 557 ± 162 U released from untreated cells (*P* < 0.01).

Possible explanations for the increased collagenase

Table 1 Collagenase activity in cell culture medium from human fibroblasts

Cells	Cell dilution* and CB addition	Cumulative collagenase during 4 d (U†)
Normal (line 236)	Zero (no CB)	658
	1:1 (CB, 1 µg ml ⁻¹)	2,967
	1:3 (no CB)	580
Normal (line 235)	Zero (no CB)	270
	1:1 (CB, 1 µg ml ⁻¹)	3,234
	1:3 (no CB)	413
Hurler	Zero (no CB)	1,455
	1:1 (CB, 1 µg ml ⁻¹)	5,357
	1:3 (no CB)	1,805

The number of cells initially seeded into the dishes did not change the collagenase activity markedly, but the additional of CB (1 µg ml⁻¹) produced a large increase with all three types of cells. See text and the legend to Fig. 1 for experimental details and assay conditions.

* Disaggregated cells (2 × 10⁵ cells per ml in DMEM with 10% FCS) were plated in duplicate plastic dishes in the dilution shown. When the dishes with the lowest dilution had reached confluence, serum-free medium was added to each dish and CB was added only to those dishes plated originally at 1:1 dilution.

† 1 Unit = 1 µg collagen degraded per h per mg cell protein.

activity in the medium brought about by CB are numerous, and include increased release of preformed collagenase already stored intracellularly, activation of a zymogen of the enzyme, destruction or blockage of an inhibitor of collagenase secreted by the cells, and (or) an increased *de novo* synthesis of collagenase. The following experiment, one of several essentially similar experiments, was carried out to show whether untreated cells had more enzyme stored intracellularly than treated cells, and to test the dependence of collagenase release on new protein synthesis. Six dishes were inoculated with rabbit synovial cells (twelfth passage); three of these were exposed to CB (1.0 µg ml⁻¹). Cycloheximide (Sigma) was added to one control and to one CB-treated dish after collection of medium on day 2 of culture. After medium was collected on day 4, the cells of each dish were washed, dispersed and a portion was counted. The remaining cell suspension was frozen and thawed rapidly (×4) and assayed for collagenase, as was the culture medium from each dish. Table 2 shows clearly that cycloheximide diminished the collagenase production in the treated cells (by more than 90%); enzyme activity in the medium from control cultures was very weak and too near baseline values to make comparisons among dishes. Little difference was found in collagenase activity between control and CB-treated cell lysates.

There is evidence that latent collagenase in culture medium from fibroblasts can be activated by incubation

with small amounts of trypsin⁵. Whether this signifies the presence of a zymogen, such as that reported for the collagenase in tadpole tails⁸, mouse bone⁹ and polymorphonuclear leukocytes^{10,11}, or destruction or saturation of inhibitory substances^{12,13} is not known. To test the possibility that CB activated a zymogen or inactivated inhibitors in these cultures, media pools from six CB and six control cultures of rabbit synovial fibroblasts (fourteenth passage) were incubated with crystalline trypsin (0.5 µg trypsin per 8.5 µg medium protein) for 1 h at 37 °C. An excess of soy bean trypsin inhibitor was then added and aliquots of each sample were assayed in quadruplicate. The following results (expressed as c.p.m. ¹⁴C-collagen solubilised per 10 h per ml) were obtained: control (no trypsin), 3.4; control (trypsin), 4.1; CB (no trypsin), 870; CB (trypsin), 1,207. Viscometric analysis (27 °C) of the trypsin-activated CB medium with subsequent electrophoresis on polyacrylamide gels revealed typical cleavage of soluble collagen into A (75%) and B (25%) fragments. Similar data have been obtained in other experiments, and we suggest, therefore, that at least a part of the collagenase activity stimulated by CB may be latent in a zymogen or inhibited form.

The precise mechanism by which CB stimulates the production of both active and latent collagenase is unknown, but our results support the idea that the important determinants which, when triggered, activate collagenase production and release by cells are located in cell membranes. Studies underway in our laboratory showing that aminophyllin stimulates collagenase release from fibroblasts (unpublished results of E. Cartwright, E. D. H. and J. J. R.) may implicate cyclic nucleotides in the control of the synthesis and release of non-lysosomal enzymes. Whatever the precise mechanism of action of CB, its use with human and animal cell cultures will be valuable for investigating the role of collagenases in connective tissue diseases.

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Table 2 Effect of cycloheximide on stimulation of collagenase by CB

		Collagenase in medium (U*)		Collagenase in cell lysate (U*)
		Days 0-2	Days 2-4	
Control cells	1	107	234	387
	2	49	87	181
	3	140	98†	157‡
CB-treated cells	1	2,018	2,196	372
	2	2,150	1,916	270
	3	2,506	226†	160‡

Rabbit cells were grown for 4 d with or without CB, and one dish from each group was also treated with cycloheximide (10 µg ml⁻¹) during the second 2-d culture period. Cell lysates were prepared at the end of the 4-d period.

* 1 Unit = 1 µg collagen degraded per 10 h per 10⁶ cells.

† Cycloheximide during days 2-4 of culture. Cycloheximide (1-100 µg ml⁻¹) had no effect on collagenase activity when added directly to assays.

‡ Cell lysates from cells to which cycloheximide had been added during culture days 2-4.

Induction of synthesis of bacterial protein by excretory product of the alga *Chlamydomonas reinhardtii* y-1

MANY examples are known of interspecies relationships between widely different organisms in natural communities¹. The benefits that one or more members of a population obtain as a result of their association have been described

mainly in terms of the nutritional value of the relationship, measured in terms of growth rate, metabolic activities, or the ability to survive in a selective environment¹⁻³. We have discovered an apparent commensal relationship between the alga *Chlamydomonas reinhardtii* y-1 and a bacterium, tentatively identified as a coryneform bacterium. As a result of their association, the synthesis of a single protein is stimulated to a remarkable extent within the bacterial cells, whereas the algal cells apparently obtain neither benefit nor detriment from the relationship.

This discovery was facilitated by the observation that ³H-leucine (40–60 Ci mmol⁻¹), when added to mixed cultures of these two organisms to a concentration near 10⁻⁷ M, is selectively incorporated only into proteins synthesised within the bacterial cells. Figures 1 and 2 show the electrophoretic patterns of radioactivity obtained after ³H-leucine was added to pure, dark-grown cultures of the bacteria (Fig. 1a) and *C. reinhardtii* y-1 (Fig. 1b), and to a mixed, dark-grown population of both organisms (Fig. 2) after 1 h of exposure to light. Very little radioactivity was

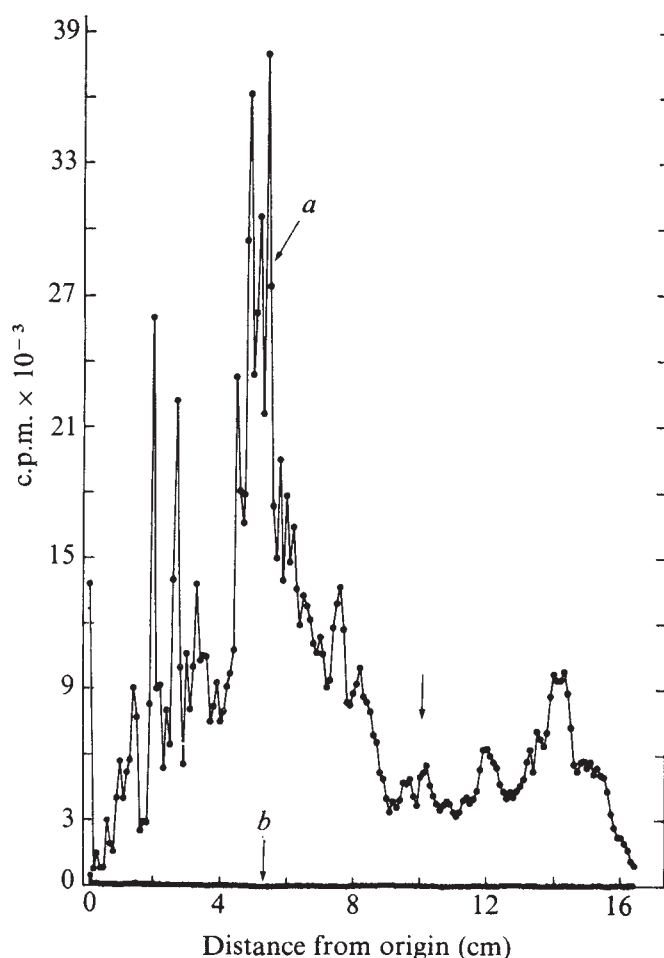


Fig. 1 Electrophoretic analysis of the incorporation of ³H-leucine into total polypeptides of bacteria (a) and *C. reinhardtii* y-1 (b), each grown separately in pure culture. Bacterial cells were grown in the dark in the algal medium⁸ containing 0.5% yeast extract and 0.5% peptone. The cells were washed twice and resuspended in the algal medium to a density of 0.14₄₅₀. Dark-grown cells of both organisms were exposed to light for 1 h, and ³H-leucine (4 μCi ml⁻¹) was added to each culture. After 1 h incubation cells from each culture were collected by centrifugation. Bacterial cells were incubated for 45 min in 25 mM Tris-HCl (pH 7.6) containing 1 mM EDTA, lysozyme 0.25 mg ml⁻¹, and chloramphenicol 25 μg ml⁻¹. In these conditions the bacterial cells swell but do not lyse. The cells were collected by centrifugation and frozen. Procedures for electrophoresis of total cellular proteins and for determination of the patterns of radioactivity were as described¹²⁻¹⁴.

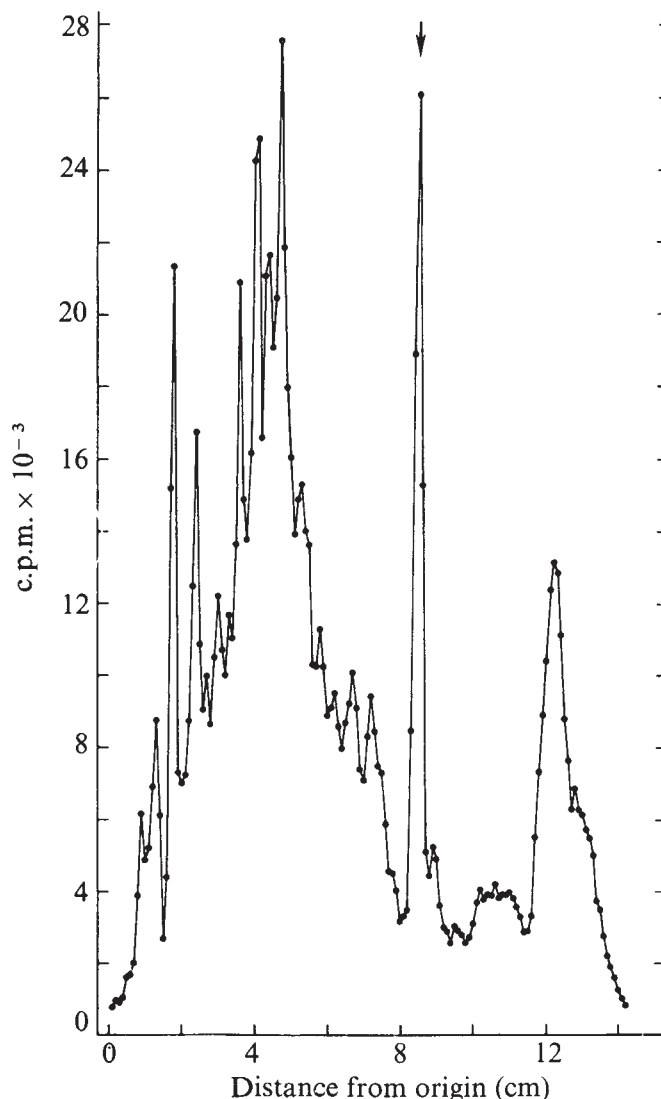


Fig. 2 Electrophoretic analysis of the incorporation of ³H-leucine into total polypeptides of a mixed population of bacterial and algal cells labelled in the light. Bacteria were grown as described in Fig. 1 and mixed with etiolated cells of *C. reinhardtii* y-1. The mixed population was exposed to light for 1 h, and incubated for an additional 1 h with ³H-leucine (4 μCi ml⁻¹) in the light. Cells of both organisms were incubated with lysozyme and total proteins were prepared for electrophoresis as described in Fig. 1.

detected in gels containing total proteins of the algal cells grown in pure culture (Fig. 1b). With the exception of a single polypeptide fraction (arrows), however, the pattern of total radioactivity for the bacterial cells alone (Fig. 1a) resembled that for mixed cells (Fig. 2). This incorporation of leucine by the bacterial cells was resistant to the action of cycloheximide, but was nearly abolished by inhibitors of 70S ribosomes⁴. Furthermore, the incorporation was blocked by inhibitors of prokaryotic RNA synthesis⁴.

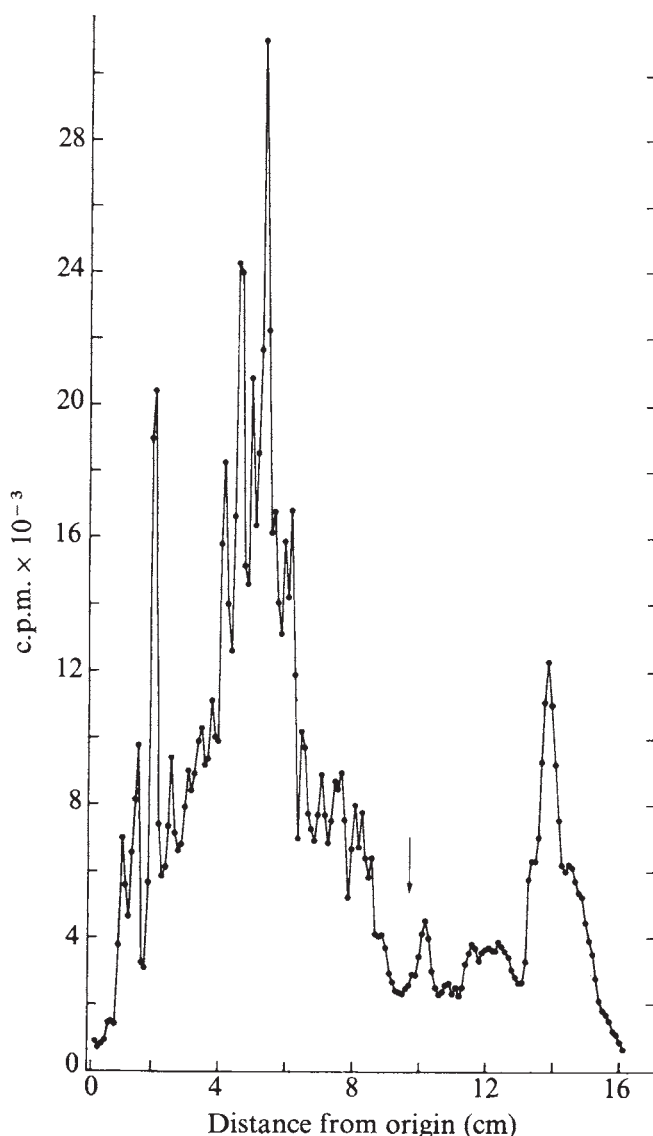
Leucine seems to be poorly transported across the plasma membrane of *C. reinhardtii*, as a several hundredfold increase in the extracellular concentration of this amino acid was required to obtain significant labelling of the algal proteins⁴. The resulting pattern of radioactivity for algal cells labelled with ³H-leucine (1 Ci mmol⁻¹, 3 × 10⁻³ M in the medium) bore no resemblance to the bacterial pattern shown in Fig. 1a.

The prominent peak of radioactivity in the pattern for mixed cultures (Fig. 2, arrow) corresponds to a polypeptide

with a molecular weight of about 21,500. Synthesis of this polypeptide within the bacterial cells was observed when dark-grown cultures of a mixed population of bacterial and algal cells was exposed to light (Fig. 2), but was not observed when a mixed culture was labelled in the dark (Fig. 3) without exposure to light. Nor was this polypeptide synthesised when dark-grown bacterial cells were exposed to light in the absence of the algal cells (Fig. 1a), suggesting that the photoreceptor for synthesis of this polypeptide resides within the algal cells. Thus, the absorption of light by etiolated cells of *C. reinhardtii* y-l elicits a response that stimulates the synthesis of a single protein within this bacterium as the result of their association.

Algae are known to excrete a wide variety of substances⁵⁻⁷, and commensal relationships have been reported in which the growth of bacteria depended on the excretion of oxidisable substrates by algal cells⁸. To test for excretion of an inducing substance by *C. reinhardtii* y-l, a pure culture of dark-grown algal cells was exposed to light, and after 90 min of illumination, the algal cells were removed by centrifugation. Synthesis of the inducible protein was

Fig. 3 Electrophoretic analysis of the incorporation of ³H-leucine into total polypeptides of a mixed population of bacterial and algal cells labelled in the dark. Dark-grown cells of both organisms were mixed and incubated with ³H-leucine (4 μ Ci ml⁻¹) for 1 h without exposure to light. After treatment with lysozyme, total proteins of the cell mixture were prepared for electrophoresis as described in Fig. 1.



stimulated when the bacteria were suspended in this medium. No induction of the synthesis of this protein, however, was detected when the bacteria were suspended in medium obtained in the same way from a mixed population of the two organisms. These results indicated that in response to light the etiolated algal cells released a limited amount of the inducing factor into the medium, and that this factor was quantitatively used by the bacterial cells.

Although we have not identified this factor, its production may be related to the breakdown of starch in etiolated *C. reinhardtii* y-l. In this alga, starch breakdown requires continuous illumination and is maximal during the lag phase of chlorophyll synthesis⁸. The time course of synthesis of the inducible protein within the bacteria during continuous illumination of mixed cultures also reached a maximum during the lag phase of chlorophyll synthesis. Thereafter, the rate declined until synthesis of this protein was barely detectable when the algal cells had become fully green (data to be published elsewhere).

The coryneform bacteria contain a large number of plant saprophytes found in soil and water environments⁹⁻¹¹. Many species within this group require vitamins and other essential organic molecules that are normally obtained from their natural habitats⁹⁻¹¹. The coryneform bacteria used in these studies were isolated as contaminants of our y-l strain of *C. reinhardtii*. They grow poorly in the defined algal medium containing acetate as a carbon source⁸, and on agar they form transparent colonies not visible to the unaided eye.

The bacteria could not be separated from the algal cells by sucrose gradient or differential centrifugation techniques⁴, because of aggregation of the bacteria in liquid media. Lysozyme treatment was required to lyse the bacteria sufficiently to release proteins for gel electrophoresis. We previously concluded that leucine had selectively entered the mitochondrial pool of amino acids within the algal cells, as the latter do lyse in these conditions without lysozyme treatment⁴.

As we do not know of any benefit derived from synthesis of the inducible protein within the bacteria, at present their relationship with *C. reinhardtii* y-l must be considered commensal only in so far as one member seems to be affected by the association. True commensalism has been found between bacteria and other algal and yeast cells based on the nutritional requirements of the bacteria^{2,3}. This is the first report, however, that such an interaction may control specific gene expression within one member of the association. Further studies are in progress to determine the identities of the algal induction factor and the protein synthesised by these bacteria in response to this factor.

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Kinetics of inactivation of histone mRNA in the cytoplasm after inhibition of DNA replication in synchronised HeLa cells

In many eukaryotic cells DNA replication and histone synthesis proceed synchronously¹⁻⁶. Interruption of DNA synthesis in synchronised HeLa cells results in an immediate decrease of histone synthesis as measured by incorporation of labelled amino acids into histones^{2,3} or by following the completion of nascent histone polypeptide chains on polyribosomes *in vitro*⁵.

Recent long-term labelling experiments by Perry and Kelley⁷ suggest that in mouse L cells the lifetime of histone mRNA is of the order of the length of one S-phase (that is about 11 h). If, however, DNA replication is blocked with cytosine arabinoside or hydroxyurea, labelled histone mRNA selectively disappears from polyribosomes between 30 and 60 min after addition of these drugs to the cells⁵⁻⁷. In contrast, actinomycin D (5 $\mu\text{g ml}^{-1}$) only slightly decreases histone synthesis during the first hour of its action⁸.

Recently we have introduced a method of titrating the amount of biologically active histone mRNA by its transla-

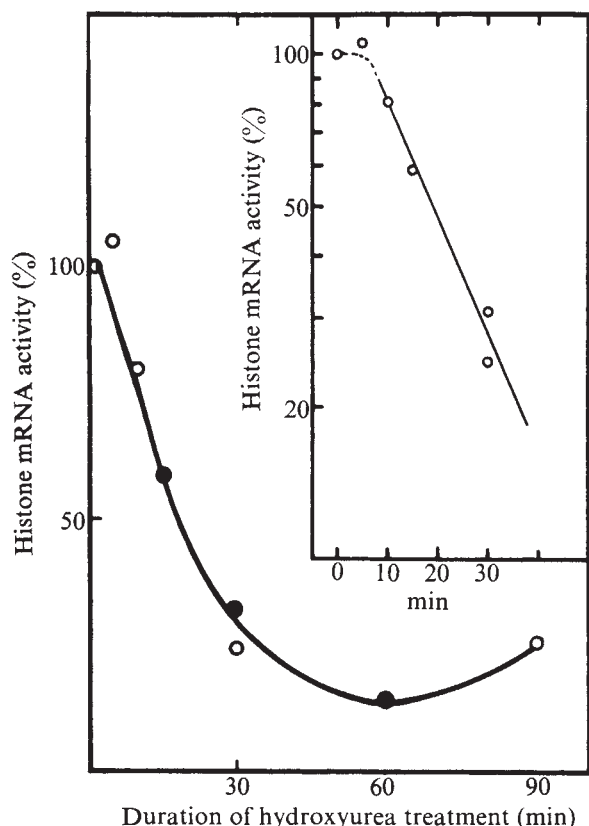


Fig. 1 Inactivation of polyribosomal histone mRNA after termination of DNA synthesis. Thymidine-synchronised cells 3 h in S-phase were treated with 3 mM hydroxyurea for different times and lysed in hypotonic buffer. Polyribosomes were isolated as described¹⁰, resuspended in 35 mM Tris-HCl (pH 7.5), 10 mM EDTA, 0.1 M NaCl, 0.5% sodium dodecyl sulphate and the RNA was extracted with buffer-saturated phenol and phenol-chloroform (1:1, v/v)¹⁰. The RNA was passed over poly(U)-Sephadex equilibrated with 10 mM Tris-HCl (pH 7.5) containing 0.5 M KCl. RNA not adsorbed to poly(U)-Sephadex was fractionated on sucrose gradients and histone mRNA translated in a rabbit reticulocyte lysate as described¹⁰. The amount of mRNA was calculated from the radioactivity incorporated into protein according to the equation $(A-B)/a$; A and B being (c.p.m. in CM-2 $\times 100$)/(c.p.m. in CM-1) from lysates incubated in the presence or absence of histone mRNA, respectively; a being (μg of polyribosomal poly(A)(-)-RNA from which histone mRNA was recovered). Open and closed circles represent data from two separate experiments. The insert is a semi-logarithmic plot of the data.

tional capacity in a rabbit reticulocyte lysate⁸. Using this method we showed that in S-phase of synchronised HeLa cells about 80% of functional histone mRNA was lost from polyribosomes within 60 min of inhibition of DNA synthesis whereas actinomycin D for 1 h only slightly decreased histone mRNA activity⁹. We describe experiments here showing that these RNA species decay from the cytoplasm with an approximate half life of 13 min after interruption of DNA replication with hydroxyurea.

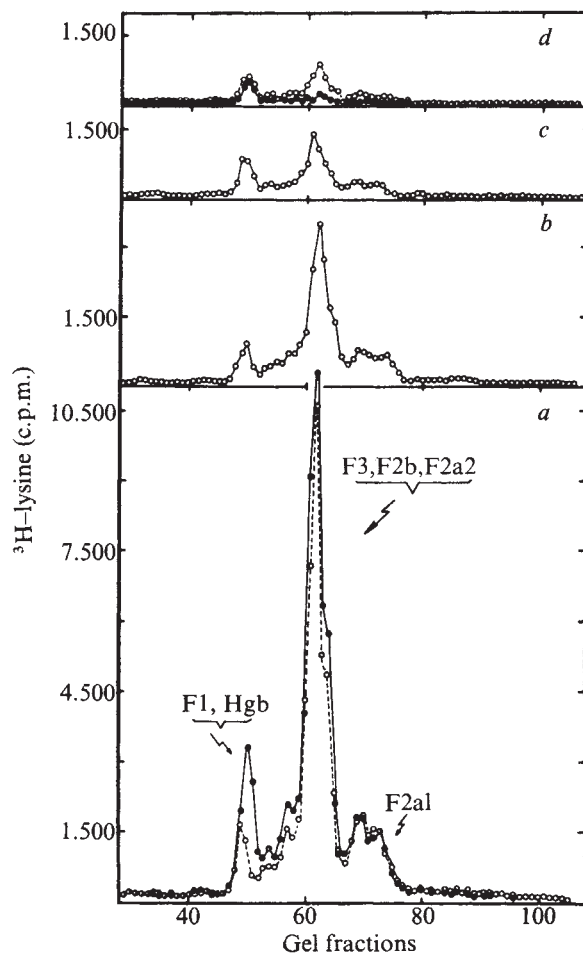


Fig. 2 Polyacrylamide gel electrophoresis of acid-soluble proteins (CM-2 fraction) synthesised in a reticulocyte lysate under the direction of histone mRNA from cells 3 (○) and 4 (●) h in S-phase (a) and from 3-h S-phase cells treated with 3 mM hydroxyurea for 15 min (b), 30 min (c) and 60 min (d, ○). CM-2 proteins from a lysate incubated without added mRNA are represented in d (●). Proteins were dissolved in 6 M urea containing 0.5 M 2-mercaptoethanol, incubated for 30 min at room temperature and electrophoresed in 15% acrylamide gels containing 2.5 M urea¹⁴. Gels were sliced and radioactivity measured as described¹⁰.

HeLa S-3 cells were grown in suspension culture in Eagle's minimum essential spinner medium supplemented with 5% calf serum. Cells were synchronised by a double block with 2 mM thymidine and allowed to enter S-phase by resuspension in fresh medium at a concentration of 3×10^5 – 4×10^5 cell ml^{-1} (ref. 10). Three and four hours after entering into S-phase the cells synthesise histones at maximal rate⁵. Cells were then treated for different times with 3 mM hydroxyurea which results, within less than 5 min, in an inhibition of thymidine incorporation by more than 98%. Polyribosomal RNA was isolated and passed over poly(U)-Sephadex columns to purify it from poly(A)-containing mRNA. Histone mRNA lacking poly(A) at the 3'-OH end¹¹ was then separated on sucrose gradients and quantitated by its translation in a reticulocyte lysate as described previously^{8,9}. Acid-soluble proteins of the lysate were separated by CM-cellulose chromatography into

two fractions, CM-1 which predominantly contains globin and CM-2 containing the histones¹⁰.

From Fig. 1 it can be deduced that as early as 10 min after a blockade of DNA replication about 20% of functional histone mRNA is lost from polyribosomes in comparison to the mRNA content of 3-h S-phase cells. Thirty and 60 min after arrest of DNA synthesis less than 30% and 15% respectively of active histone mRNA are left on polyribosomes. Further treatment with the blocking agent did not change this value significantly. A further increase of polyribosomal histone mRNA by about 20–30% was observed between the third and fourth hour after entry into S-phase which subsequently decreased again (data not shown). It should be pointed out that active histone mRNA does not survive unbound to ribosomes in the cytoplasm after DNA synthesis is stopped^{9,12}.

The gel electrophoretic profiles show that synthesis of all histone fractions is equally inhibited following the block of DNA replication (Fig. 2). It can also be seen in Fig. 2 that CM-2 proteins from reticulocyte lysates incubated without added mRNA contain labelled material which predominantly coelectrophoreses with globin (Fig. 2d, ●, fractions 48–52). Therefore, such control lysates had to be taken into account in the calculation of histone mRNA activity (see legend to Fig. 1).

If it is assumed that after interruption of DNA replication the flow of new histone mRNA from the nucleus to the cytoplasm immediately ceases (as a result of an inhibition of either the transcription of histone genes, the processing of histone mRNA in the nucleus or its transport into the cytoplasm) the disappearance of histone mRNA from the cytoplasm is a function mainly of the degradation (or inactivation) of that RNA. From a semilogarithmic plot of the data presented in Fig. 1 it can be deduced that, after a short lag of about 5 min, histone mRNA is inactivated according to first-order kinetics with a

half life of about 13 min (insert in Fig. 1). The short lag possibly results from the time required for hydroxyurea to penetrate the cells and to interrupt subsequent flow of histone mRNA from the nucleus to the cytoplasm.

Butler and Mueller¹³ recently reported that cycloheximide prevents the inhibition of histone mRNA synthesis by hydroxyurea. We measured the polyribosomal mRNA in cells which were treated with cycloheximide and hydroxyurea simultaneously and found that although DNA replication was completely blocked by the two drugs histone mRNA survived on polyribosomes in these experimental conditions (Fig. 3). Since cycloheximide 'freezes' polyribosomal structures this study indicates that histone mRNA in cells treated in this manner is protected from degradation by its binding to ribosomes. This implies that a step in the initiation of histone mRNA translation may be inhibited in the absence of DNA replication so that the mRNA becomes susceptible to cellular nuclease(s). Alternatively, the inactivation of histone mRNA in the cytoplasm after cessation of DNA synthesis could be linked in some unknown manner to a process depending on protein synthesis.

The extremely short half life of histone mRNA in the absence of DNA replication described here is consistent with the data presented by Perry and Kelley⁷ which indicate that in mouse L cells the decay of histone mRNA increases nearly linearly with the age of these RNA species. From all the known experimental results it is now well substantiated that histone mRNA is present on polyribosomes only during the DNA replication period of the cell cycle^{5–7,10,12,15}. These RNA species are synthesised during the first few hours of S-phase starting with the initiation of DNA replication^{6,10}, they are stable during S-phase⁷ and are destroyed with a half life of about 13 min after termination of DNA synthesis. The shut-down of histone synthesis at the end of S-phase is therefore the consequence of an inactivation of cytoplasmic histone mRNA which seems to involve a specific translational control mechanism operating at the initiation of histone mRNA translation.

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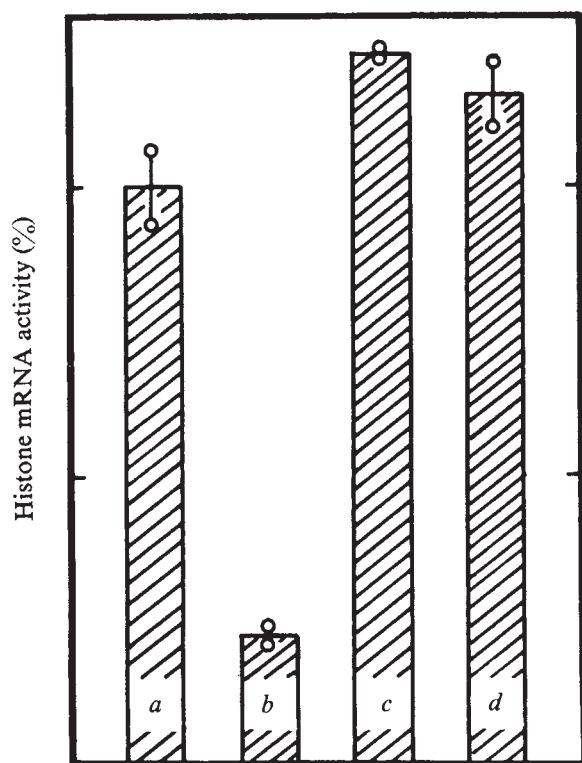


Fig. 3 Effect of cycloheximide on hydroxyurea-induced inactivation of histone mRNA. 600-ml spinner cultures of synchronised cells 3 h in S-phase were untreated (a) or treated for 45 min with either 3 mM hydroxyurea (b), 10 µg ml⁻¹ cycloheximide (c) or with both drugs simultaneously (d). In the latter case cycloheximide was added to the cultures 3 min before hydroxyurea. Two minutes before collecting the cells, cycloheximide was also added to cultures a and b. The data represent two separate experiments.

In vitro transcription of a late class of phage SP01 genes

Bacillus subtilis phage SP01 directs the synthesis of at least six temporally defined classes of phage-specified transcripts¹. Synthesis of the two earliest classes of phage RNA (e and em) does not require phage protein synthesis, while the synthesis of middle (m and m₁l) and late (m₂l and l) transcripts requires the protein product of regulatory gene 28 and the products of regulatory genes 33 and 34, respectively^{2,3}. The discovery of SP01-induced polypeptides associated with *B. subtilis* RNA polymerase suggested that the products of SP01 regulatory

genes could control transcription by interacting directly with the host RNA polymerase^{4,5}. In support of this idea, we reported⁶ that a form of RNA polymerase from SP01-infected cells copies middle RNA almost exclusively from the heavy (H) strand of native SP01 DNA, the DNA strand from which middle and late genes are transcribed *in vivo*. This enzyme contains an SP01-induced polypeptide termed IV (molecular weight 26,000) that is now known to be the product of regulatory gene 28 (T. Fox, R. L. and J. P., manuscript in preparation). Accurate middle gene transcription by this phage-modified RNA

polymerase does not require σ factor but is apparently dependent on a newly described host subunit of RNA polymerase called δ (molecular weight 21,500)⁶. Other workers have also reported the preferential synthesis of middle RNA by RNA polymerase from phage-infected *B. subtilis*^{7,8}. Here we report that in the presence of host δ protein a form of RNA polymerase containing phage-induced polypeptides termed V (molecular weight 24,000) and VI (molecular weight 13,500) asymmetrically transcribes a class of SP01 late genes *in vitro*.

Two forms of RNA polymerase designated enzymes B and C were purified from SP01-infected bacteria as previously described⁶. In addition to the subunits of core RNA polymerase (β' , β , α and ω), enzyme C contained phage-induced subunits V and VI, while enzyme B contained phage-induced subunit IV and traces of VI (Fig. 1). Both enzymes lacked the host δ protein and copied RNA with only a small preference for the H strand of native SP01 DNA (Fig. 2). In the presence of δ protein purified from uninfected bacteria (Fig. 1), however, enzymes B and C copied RNA predominantly from strand H (the H/L hybridisation ratios increased from 1.7 to 9.4 and from 1.2 to 8.6, respectively; Fig. 2). As previously reported⁶, δ had little effect on the strand selectivity of transcription by core enzyme from uninfected cells (the H/L ratio increased from 1.2 to 2.0; data not shown). Thus in the presence of δ ,

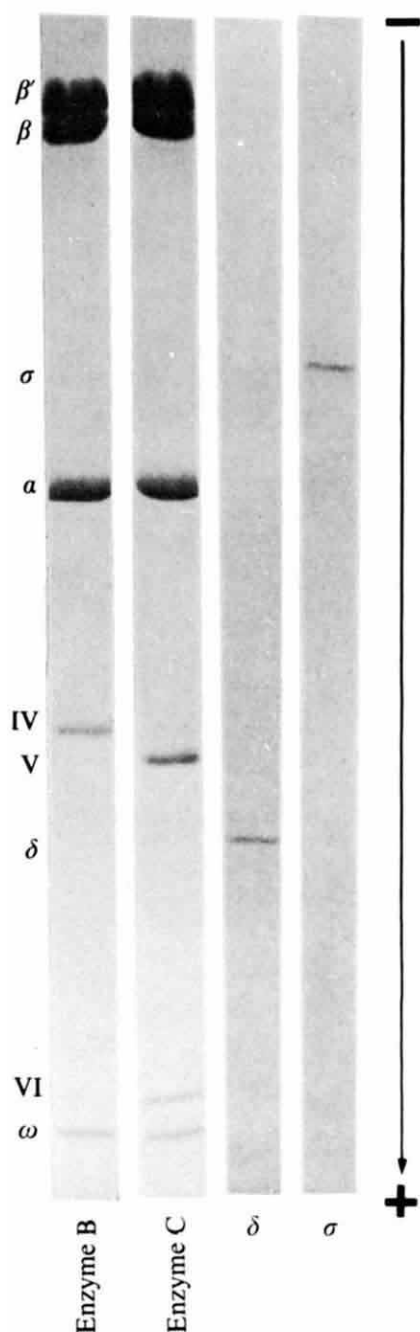


Fig. 1 SDS polyacrylamide slab gel electrophoresis of enzyme B, enzyme C, δ and σ . Enzymes B and C were purified as described previously⁶. δ and σ were dissociated from purified holoenzyme⁹ from uninfected *B. subtilis* by chromatography on phosphocellulose⁹, dialysed against buffer C (ref. 4) containing 0.01 M EDTA and 0.02 M KCl, and then applied to a poly(C)-cellulose column¹⁰. δ eluted in the flow-through while σ , which adhered to the column, was eluted with buffer containing 8 M urea. Electrophoresis was on a 25-cm long slab gel containing sodium dodecyl sulphate in a Tris-glycine buffer and a 7–15% gradient of polyacrylamide¹¹.

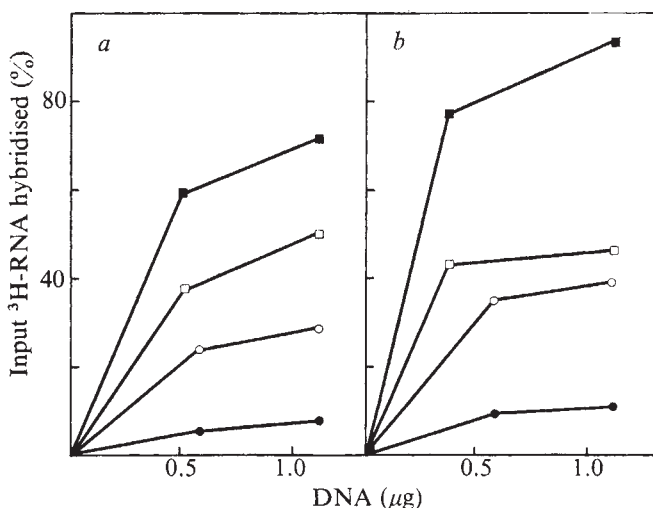


Fig. 2 Effect of δ on the strand selectivity of transcription by enzymes B and C. Radioactive RNA was synthesised *in vitro* by 3.5 μ g of enzyme B and 2.0 μ g of enzyme C in the absence (open symbols) and presence (closed symbols) of 0.4 μ g of purified δ (Fig. 1). Hybridisation reactions contained the indicated amounts of H strand ($\square$, $\blacksquare$) and L strand ($\circ$, $\bullet$) DNA and the following 3 H-RNAs in 125 μ l of $2 \times$ SSC: a, RNA synthesised by enzyme B ($\square$, $\circ$) (input, 25,200 c.p.m.; background, 490 c.p.m.); RNA synthesised by enzyme B + δ ($\blacksquare$, $\bullet$) (input, 22,700 c.p.m.; background, 1,100 c.p.m.). b, RNA synthesised by enzyme C ($\square$, $\circ$) (input, 14,500 c.p.m.; background, 540 c.p.m.); RNA synthesised by enzyme C + δ ($\blacksquare$, $\bullet$) (input, 11,700 c.p.m.; background, 280 c.p.m.). The experimental procedures for the synthesis of RNA, preparation of DNA strands and the hybridisation of RNA were described previously⁶.

enzymes B and C directed highly asymmetric transcription of SP01 DNA.

Since enzymes B and C contained different phage-induced polypeptides, it seemed possible that in the presence of host δ protein each phage-modified RNA polymerase was transcribing asymmetrically a different class of SP01 genes. To compare the classes of RNA synthesised by enzyme B + δ and enzyme C + δ , radioactive RNA synthesised *in vitro* was hybridised to denatured SP01 DNA in the presence of unlabelled RNA from cells collected at early (CAM), middle (10') and late (30') times in the lytic cycle. (The early RNA was purified from cells that had been treated with chloramphenicol to block phage protein synthesis.) The experiment of Fig. 3c shows that the hybridisation of RNA synthesised by enzyme B + δ was more effectively

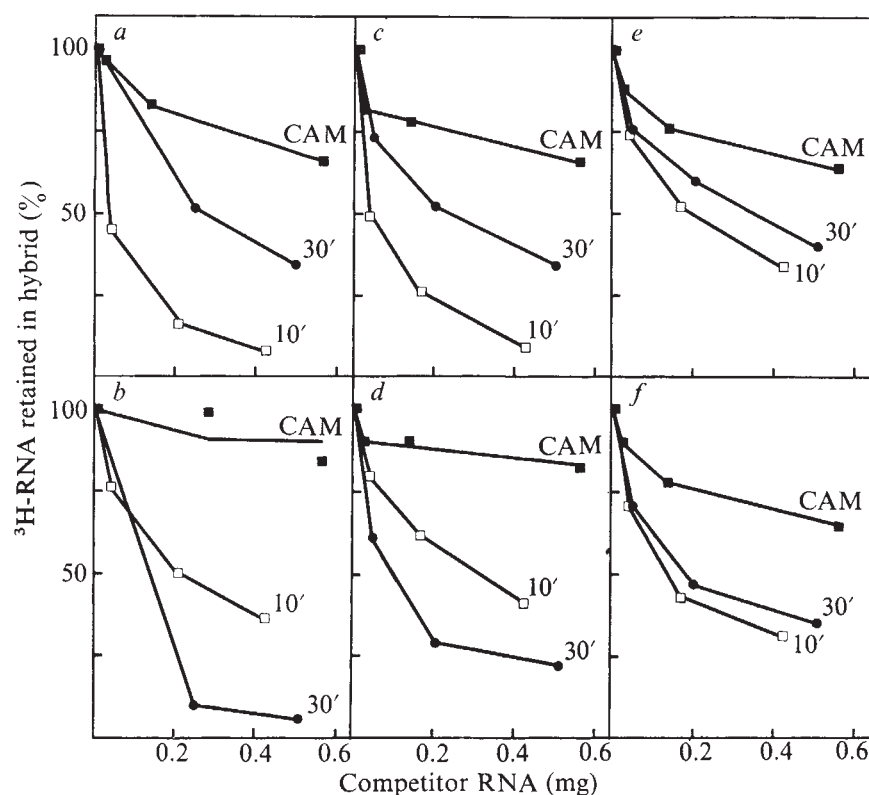


Fig. 3 Hybridisation competition of ³H-RNAs by competitor RNAs isolated at various times after infection. Hybridisation reactions contained 2.5 μ g of denatured SP01 DNA, the indicated amounts of unlabelled competitor RNAs and the following ³H-RNAs in a total volume of 200 μ l of 2 $\times$ SSC. *a*, Middle SP01 RNA pulse labelled *in vivo* 8-10 min after infection; 33% of the input RNA (21,000 c.p.m.) hybridised in the absence of competitor. *b*, Late SP01 RNA pulse labelled *in vivo* 27-29 min after infection; 18% of the input RNA (23,000 c.p.m.) hybridised in the absence of competitor. *c*, RNA synthesised *in vitro* by enzyme B+ δ ; 39% of the input RNA (9,800 c.p.m.) hybridised in the absence of competitor. *d*, RNA synthesised *in vitro* by enzyme C+ δ ; 61% of the input RNA (11,200 c.p.m.) hybridised in the absence of competitor. *e*, RNA synthesised *in vitro* by core polymerase from uninfected *B. subtilis*; 50% of the input RNA (8,100 c.p.m.) hybridised in the absence of competitor. *f*, RNA synthesised *in vitro* by enzyme C; 49% of the input RNA (11,600 c.p.m.) hybridised in the absence of competitor. For each reaction a background of less than 3% of the input was subtracted from the radioactivity that was retained in hybrid. CAM (■) RNA was from bacteria treated with 150 μ g of chloramphenicol before infection; 10' (□) RNA and 30' (●) RNA were from cells collected 10 and 30 min after infection, respectively. These competitors were prepared as described previously¹². The experimental procedure for the labelling of RNA *in vivo*, for the synthesis of labelled RNA *in vitro* and for hybridisation of radioactive RNA was as described previously⁶.

inhibited by middle RNA than by the late or early competitors. In fact, the RNA competitors similarly affected the hybridisation of *in vivo* synthesised RNA radioactively labelled at a middle time in the lytic cycle (Fig. 3a). In contrast, the hybridisation of RNA synthesised *in vitro* by enzyme C+ δ was more effectively competed by late RNA than by the middle or early competitors (Fig. 3d); and this competition pattern was similar to that obtained for RNA labelled *in vivo* at a late time in the lytic cycle (Fig. 3b). As further evidence that enzyme C+ δ was preferentially synthesising a class of late RNA, we found that RNA extracted at a late time after infection by a gene 34 mutant was a poor competitor of the *in vitro* product of enzyme C+ δ (data not shown). (This mutant phage fails to synthesise late transcripts *m₂l* and *l* (refs 2 and 3).) The specific transcription of SP01 late genes *in vitro* requires the host δ protein since enzyme C alone copied RNA symmetrically (Table 1, ref. 6) from both strands of native phage template (Fig. 2b) and since the hybridisation of RNA synthesised by enzyme C alone was not preferentially competed by late RNA (Fig. 3f). It should be noted, however, that our experiments do not distinguish whether enzyme C+ δ copies all late transcripts (*m₂l* and *l*) or only a limited set of late sequences.

Which, if any, of the phage-induced polypeptides present in enzymes B and C direct the transcription of middle or late genes *in vitro*? Enzyme B contains phage-induced subunit IV and traces of subunit VI. It is likely that subunit IV causes the synthesis of middle transcripts (*m* and/or *m₁l*) *in vitro* since polypeptide IV is now known to be coded by gene 28 (T. Fox, R. L. and J. P., manuscript in preparation), the regulatory gene whose product is necessary for middle gene transcription *in vivo*^{2,3}. While polypeptide IV is synthesised early in the phage lytic cycle, as would be expected of a protein that directs middle gene transcription, polypeptides V and VI first appear at a middle time in the lytic cycle (ref. 4 and T. Fox, unpublished results), a finding consistent with the idea that either or both of these proteins could direct late transcription by enzyme C. A reconstitution experiment with the isolated subunits IV, V and VI will, however, be required to prove that these phage-induced proteins direct specific gene transcription.

Escherichia coli RNA polymerase purified from phage T4-infected bacteria also contains phage-induced polypeptides¹³. Two of these proteins are coded by T4 genes 33 and 55, regulatory genes whose products direct late T4 transcription *in vivo*^{14,15}. Purified *E. coli* RNA polymerase containing these phage-induced subunits does not, however, specifically direct late transcription *in vitro*¹⁶. This could result from the apparent coupling of late transcription to DNA synthesis *in vivo*^{17,18}. It is also possible, however, that late transcription by the T4-modified polymerase requires an unidentified *E. coli* protein equivalent to *B. subtilis* δ in addition to T4 regulatory proteins.

In conclusion, the finding of specific transcription of SP01 middle and late genes by RNA polymerase containing phage-induced subunits supports a model of positive control elaborated by Travers¹⁰ in which phage regulatory proteins direct specific transcription by interacting with RNA polymerase in place of the host σ factor.

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N⁶, O^{2'}-dimethyladenosine a novel methylated ribonucleoside next to the 5' terminal of animal cell and virus mRNAs

MODIFIED 5'-terminal structures of the type m⁷G(5')ppp(5')Nm have been identified in various viral^{1–4} and cellular^{5–8} mRNAs. The 5'-terminal modification of mRNA may be carried out by specific guanylyl- and methyltransferases⁹ and the structure seems to be required for efficient *in vitro* translation¹⁰. Studies⁵

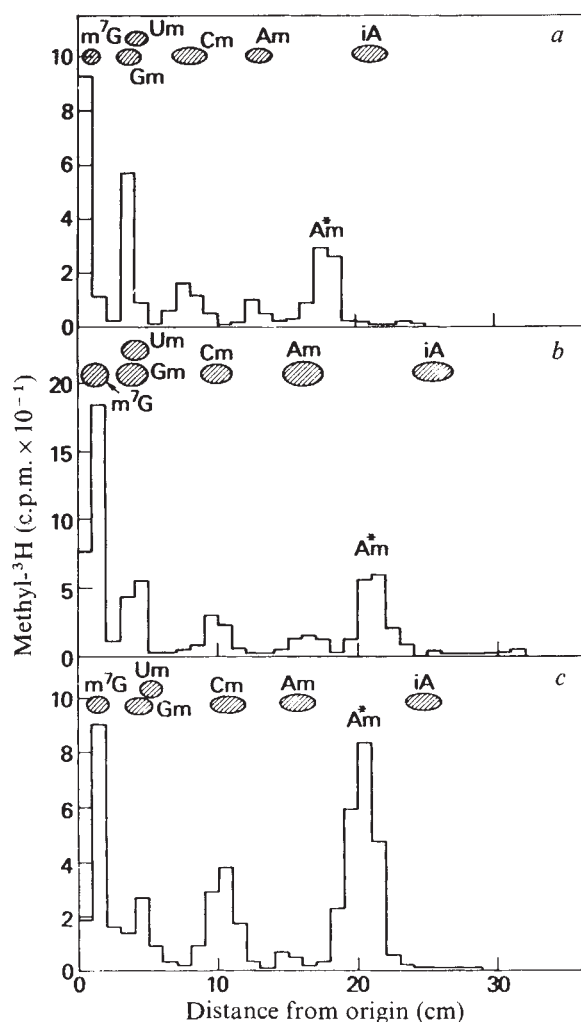


Fig. 1 Paper chromatography of methylated ribonucleosides from 5'-terminal oligonucleotides of: *a*, HeLa cell mRNA; *b*, mouse L cell mRNA; and *c*, adenovirus mRNA. Cells were labelled for 4 h with methyl-³H methionine, and polyadenylated mRNA was isolated from the cytoplasm by poly(U)-Sepharose chromatography as previously described⁵. Polyadenylated mRNA from the cytoplasm of adenovirus-infected HeLa cells was also selected by hybridisation to adenovirus DNA (B.M. and F. Kocot, unpublished). After hydrolysis of HeLa and L cell mRNAs with RNase T2, T1 and A and adenovirus mRNA with 0.4 N KOH the 5'-terminal oligonucleotides were isolated by DEAE-cellulose chromatography as the –5 to –6 charge peak⁵. The desalted 5'-terminal oligonucleotides were then digested to their constituent ribonucleosides by a combination of venom phosphodiesterase and alkaline phosphatase. The digest was analysed by ascending paper chromatography with solvent containing *n*-butanol-concentrated NH₃-H₂O (86:5:14).

of the 5'-terminal sequences of HeLa cell mRNAs revealed a methylated nucleoside that chromatographed slightly ahead of 2'-O-methyladenosine (Am). We have now identified this novel nucleoside and found that it is common to mRNAs of human and mouse cells as well as at least one virus.

The 5'-terminal oligonucleotides obtained by alkali or RNase digestion of HeLa cell mRNAs have the structures m⁷G(5')ppp(5')NmpNp and m⁷G(5')ppp(5')NmpNmpNp and elute from DEAE-cellulose columns with net charges of –5 to –6 (refs 5 and 7). Figure 1 shows a nucleoside analysis of the 5'-terminal oligonucleotides from methyl-³H-methionine-labelled mRNAs of HeLa cells, mouse L cells and adenovirus grown in HeLa cells. In addition to 7-methylguanosine (m⁷G) and the four common 2'-O-methylribonucleosides (Gm, Um, Cm and Am), a major component of each mRNA chromatographed between Am and N⁶-isopentenyladenosine (iA). The unusual nucleoside was designated A*m and tentatively considered to be a derivative of adenosine since it comigrated on electrophoresis with adenosine and Am in 1 M formic acid (Fig. 2). A*m was further shown to lack *cis*-hydroxyls by borate electrophoresis at pH 9 (ref. 5) and therefore seemed to be a derivative of Am with an additional base modification. The following experiment was designed to test whether the base was methylated and if so to identify it.

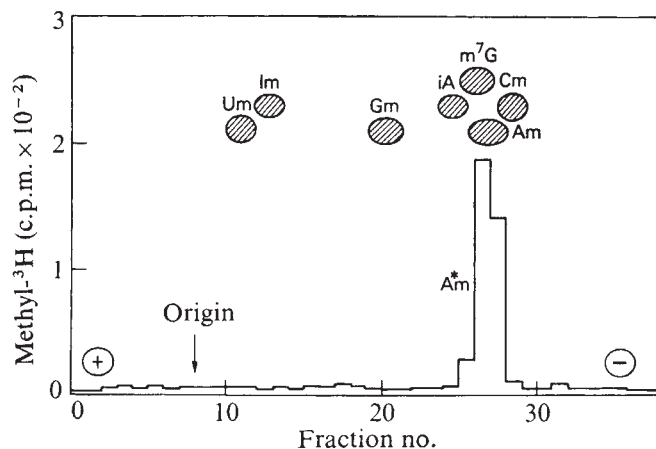


Fig. 2 Thin-layer electrophoresis of A*m. Methyl-³H-labelled A*m was isolated by paper chromatography as in Fig. 1a and analysed by electrophoresis on a thin layer of cellulose in 1 M formic acid at 800 V for 70 min.

The 5'-terminal oligonucleotides of HeLa cell mRNA were isolated and digested with P₁ nuclease (an enzyme that hydrolyses RNA to 5'-nucleotides and is not inhibited by 2'-O-methylribonucleosides¹¹) and nucleotide pyrophosphatase (an enzyme that cleaves m⁷GpppNm to pm⁷G, pNm and Pi²) to liberate methyl-labelled pA*m. The latter comigrated with pA on paper electrophoresis at pH 3.5, further suggesting that the novel compound is a derivative of adenosine. The isolated pA*m along with a small amount of contaminating pAm was then treated with 1 N HCl at 100 °C to liberate the free base. Slightly more than half of the methyl-labelled material co-chromatographed with N⁶-methyladenine (m⁶Ade) while the remainder, presumably the degradation product of 2'-O-methylribose phosphate, stayed at the origin (Fig. 3a). Since no alkaline step was used in this procedure it is unlikely that m⁶Ade was derived by rearrangement of m¹Ade. Some apparent loss of the 2'-O-methylribose derivative resulted from the lability of the O-methyl bond in 1 N HCl at 100 °C. As a control experiment, methyl-labelled pAm was isolated from the 5'-terminus of vaccinia virus mRNA¹ and treated similarly. The methyl-labelled material remained at the origin and none chromatographed as m⁶Ade (Fig. 3b).

Further evidence that the modified base of A*m is m⁶Ade was obtained by labelling HeLa cell mRNA with 2,8-³H-adenosine. Combined P₁ nuclease and alkaline phosphatase digestion followed by adsorption of the enzyme-resistant

material to a DEAE-cellulose column was used to separate the 5'-terminal oligonucleotide containing A*m from all but a small amount of the thousandfold or greater excess of internal adenosine residues. Nucleoside analysis indicated incorporation of radioactive isotope into A*m (Fig. 4a). The purified 2,8-³H-A*m was then treated with 1 N HCl at 100 °C and the labelled base released was identified as m⁶Ade in three different chromatographic systems, one of which is shown in Fig. 4b.

The sugar moiety of A*m was also characterised by cleaving methyl-³H-A*m with spleen purine nucleoside phosphorylase (Sigma) in the presence of phosphate. The labelled product cochromatographed, on a thin-layer cellulose sheet in ethyl acetate, isopropanol, 7.5 M NH₄OH, *n*-butanol (3:2:2:1), with 2'-O-methylribose-1-phosphate derived from authentic Am (not shown). In the latter experiment radioactivity was lost from the methylated base because of the deaminase activity associated with nucleoside phosphorylase¹².

We conclude that the novel nucleoside found in HeLa cell, L cell and adenovirus mRNAs is N⁶,O^{2'}-dimethyladenosine (m⁶Am). Furthermore, m⁷G(5')ppp(5')m⁶Am is the major 5'-terminal sequence of the type m⁷G(5')ppp(5')Nm in both HeLa cell mRNAs, where it comprises about 20–30% of the total (C.-M.W., A.G. and B.M., unpublished), and adenovirus mRNAs, where it comprises 80% of the total (B.M. and F. Koczot, unpublished). In addition both Adams and Cory⁶ studying mouse myeloma cell mRNA, and Moyer *et al.*¹³ studying vesicular stomatitis virus mRNA synthesised *in vivo* noted an unidentified nucleoside adjacent to m⁷G which they suspected was a derivative of Am with a modified base. By contrast m⁶Am is definitely not present in either vaccinia virus¹ or vesicular stomatitis virus⁴ mRNAs synthesised *in vitro* by virion-associated enzymes, suggesting that a cellular methyltransferase is used. This methyltransferase must be quite specific since m⁶Am has not been identified in either tRNA or rRNA. A recent report¹⁴, however, indicates that an internal

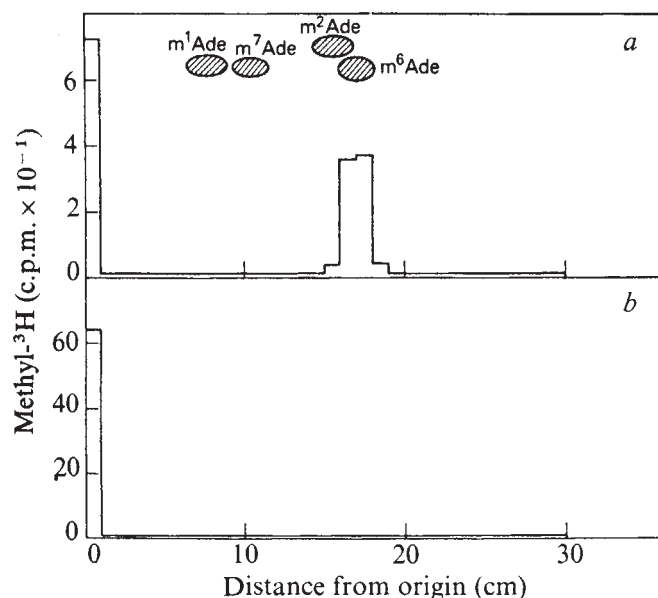


Fig. 3 Identification of methylated base. *a*, Methyl-³H-labelled 5'-terminal oligonucleotides of HeLa cell mRNA were isolated as in Fig. 1a and successively digested with 100 µg of nuclease P₁ in 200 µl of 10 mM sodium acetate; pH 6, at 37 °C for 1 h and 0.2 U nucleotide pyrophosphatase in 20 mM Tris-HCl, pH 7.6, 1 mM MgCl₂ at 37 °C for 30 min. The ribonucleotides were separated by electrophoresis on a 40 cm sheet of Whatman 3 MM paper in 50 mM ammonium formate, pH 3.5, 1 mM EDTA at 1,900 V for 3 h. The fractions corresponding to adenylic acid marker and containing pA*m were pooled. pA*m was heated with 1 N HCl at 100 °C for 30 min and the product was analysed by ascending paper chromatography with solvent containing *n*-butanol-concentrated NH₃-H₂O (86:5:14). *b*, Methyl-³H-labelled pA isolated from vaccinia virus mRNA¹ was treated with 1 N HCl and similarly analysed.

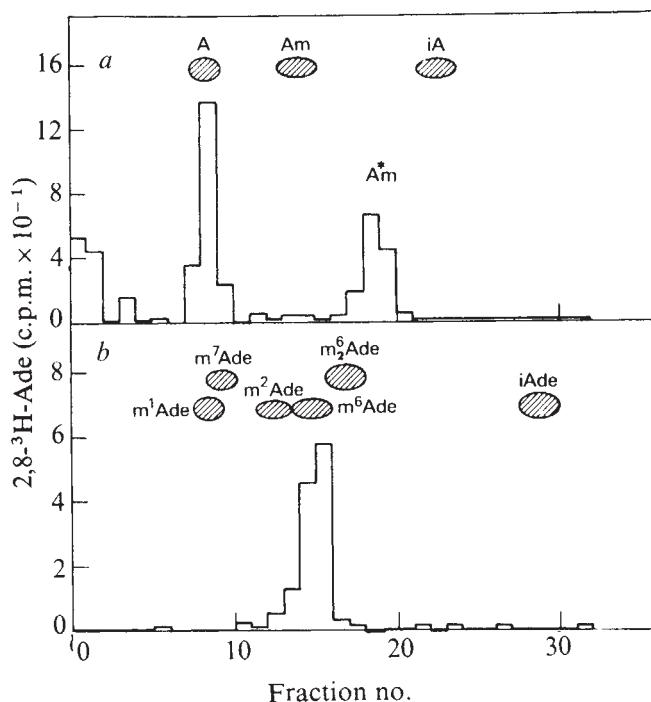


Fig. 4 Incorporation of 2,8-³H-adenine into N⁶-methyladenine moiety of A*m. *a*, HeLa cells (4 × 10⁸) were incubated in 1 l of medium containing 20 µM guanosine and 10 mCi (40.8 Ci mmol⁻¹) of 2,8-³H-adenosine for 24 h at 37 °C. The cytoplasmic polyadenylated mRNA was isolated as in Fig. 1 and then digested with nuclease P₁ (50 µg per 100 µl) in 10 mM sodium acetate, pH 6, at 37 °C for 1 h and then with alkaline phosphatase (10 µg per 100 µl) in 50 mM Tris-HCl, pH 8.5, 5 mM MgCl₂ at 37 °C for 2 h. The diluted digest was applied to a 5 ml DEAE-cellulose column equilibrated with 25 mM ammonium acetate and then extensively washed with 25 mM ammonium acetate. The labelled m⁷G(5')ppp(5')Nm was eluted with 1 M ammonium acetate, lyophilised and digested with venom phosphodiesterase and alkaline phosphatase. The nucleosides were analysed by ascending paper chromatography with solvent containing *n*-butanol-concentrated-NH₃-H₂O (86:5:14). *b*, Identification of 2,8-³H-N⁶-methyladenine. Labelled A*m isolated by paper chromatography as indicated above (*a*) was treated with 1 N HCl at 100 °C for 30 min and analysed by thin-layer chromatography with solvent containing isopropanol-concentrated HCl-H₂O (680:144:170).

m⁶Am residue is present in a low molecular weight nuclear RNA. It is possible that m⁶Am has a role in protein synthesis since in mRNAs it lies adjacent to the m⁷G residue that is required for efficient translation of certain mRNAs *in vitro*¹⁰.

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Two distinct mechanisms of synthesis of DNA fragments on colicin E1 plasmid DNA

REPLICATION of closed-circular colicin E1 plasmid (Col E1) DNA can be initiated and completed in extracts of *Escherichia coli*^{1,2}. The major products of *in vitro* replication are completely replicated molecules (23S) and a unique type of early replicative intermediate (26S) containing a newly synthesised DNA fragment(s) in a small replication loop. The fragment has an average length of approximately one fifteenth of the unit length of the plasmid molecule and has a sedimentation constant of approximately 6S^{1,3}. The replicated region of the intermediate consists of either one double-stranded branch and one single-stranded branch (type I) or two double-stranded branches (type II)^{2,4}. These intermediates accumulate in a reaction mixture containing 10% glycerol^{2,3}. Synthesis of the intermediates is inhibited by rifampicin^{1,3} but most of the intermediates can complete replication in the presence of rifampicin³. Rifampicin is also known to inhibit *in vivo* replication of Col E1 DNA⁵. We have studied the synthesis and fate of 6S DNA fragments formed on the parental heavy (H) strands and those formed on the parental light (L) strands (defined by CsCl density gradient centrifugation in the presence of poly(U,G)) of early replicative intermediates. The results show that the first synthesis of a DNA fragment is initiated at a specific site on the H strand and depends on the function of

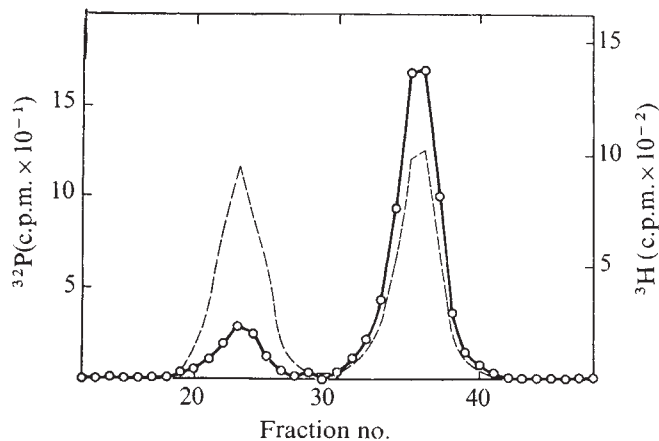


Fig. 2 Distribution of radioactivity in strands of completely replicated molecules derived from early replicative intermediates with ³²P-labelled 6S DNA. The DNA formed by incubation of sample C as described in the legend to Fig. 1b was centrifuged after heating at 90 °C for 90 s in neutral sucrose gradient (5-20%) at 45,000 r.p.m. for 150 min at 10 °C in a SW 50.1 rotor¹. Completely replicated closed-circular molecules (23S) thus isolated were converted to the linear form by treating with endonuclease *EcoRI*⁴. The linear molecules were denatured and centrifuged in a CsCl density gradient containing poly(U,G) and fractionated as described⁶. The reference DNAs were, from the left, H strands and L strands of Col E1 DNA. —, ³²P; ---, ³H.

E. coli DNA-dependent RNA polymerase. Subsequent synthesis of the DNA fragment on the L strand does not involve the RNA polymerase.

To examine whether or not the strand in the type I intermediates was specifically synthesised on one of the parental strands, early replicative intermediates with 6S fragments labelled, *in vitro*, with α -³²P-dTMP were prepared (Table 1). Samples A and B were made after incubation for 10 or 45 min at 30 °C of a reaction mixture with a freshly prepared cell extract; sample C was made from a reaction mixture incubated for 45 min with a cell extract stored for 1 week at -20 °C. After purification of ³²P-labelled replicative intermediates³, labelled 6S DNA was separated from the parental strands by heating at 90 °C for 90 s (ref. 3) and then annealed with separate H and L strands of Col E1 DNA fixed on membrane filters. Table 1 shows that in all cases more labelled 6S fragments were hybridised to H strands than L strands. Synthesis of two types of 6S fragments was more asymmetrical for the molecules labelled for 10 min than for 45 min. It was also more asymmetrical for the molecules synthesised with the stored extract than with the fresh extract. In sample B, 33% of 6S fragments was hybridisable to L strands, while about 40% of such a preparation of intermediates was type II as examined electron microscopically². These results show that most type I intermediates had a 6S L fragment.

Formation of early replicative intermediates is sensitive to rifampicin, but most of the intermediates formed in a cell extract can complete replication during continued incubation after addition of rifampicin³. These results indicate either that no further products of the rifampicin-sensitive reaction were necessary for completion of replication of the intermediates, or that products formed before addition of rifampicin could be used for the replication that took place after addition. The validity of these interpretations was tested by examining replication of exogenously added early replicative intermediates in the presence of rifampicin. The preparation of the intermediates (sample C of Table 1) used for this purpose was practically free from labelled, completely replicated molecules as shown by alkaline sucrose density gradient centrifugation of the preparation (Fig. 1a). By contrast, the labelled DNA extracted after the incubation of the intermediates in the presence of rifampicin contained approximately 55% collapsed closed-circular molecules and 10% unit-length single strands (Fig. 1b). Completely replicated molecules were formed from

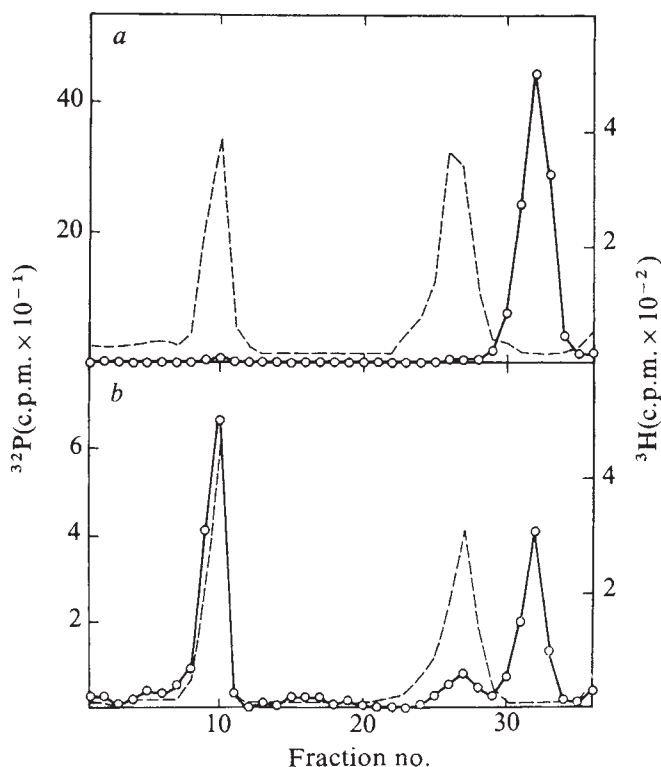


Fig. 1 Alkaline sucrose gradient centrifugation patterns of ³²P-labelled early replicative intermediates before (a) and after incubation in a cell extract with rifampicin (b). A portion of sample C (unheated) in Table 1 was centrifuged in an alkaline sucrose gradient (5-20%) at 45,000 r.p.m. for 2 h at 5 °C in a Beckman SW 50.1 rotor¹. Fractions were collected from the tube bottom. The pattern of distribution of radioactivity is shown in (a). Another portion of sample C (unheated) was incubated for 90 min at 30 °C in the standard reaction mixture¹ containing an extract of YS1 cells and rifampicin (10 µg ml⁻¹) without glycerol and spermidine. DNA was extracted and similarly centrifuged (b). The reference DNAs were, from the left, ³H-labelled collapsed closed-circular Col E1 DNA and circular and linear DNA of the unit length. —, ³²P; ---, ³H.

the early replicative intermediates also in the presence of antibody against *E. coli* RNA polymerase which inhibited formation of the intermediates (data not shown). To test whether type I intermediates replicate in the presence of rifampicin, the distribution of radioactivity between H and L strands of completely replicated molecules derived from the intermediates was examined. After conversion of the molecules to the linear form by treating with endonuclease *EcoRI*⁴, the single strands of the molecules were centrifuged in a CsCl density gradient containing poly(U,G)⁶. The labelled DNA was distributed in L and H strands at the ratio of approximately 6:1 (Fig. 2). If only the type II intermediates completed replication or if labelled DNA was degraded and labelled material was reincorporated into closed-circular molecules, the radioactivity would have been distributed almost equally between the complementary strands of the molecules. The unequal distribution of radioactivity between the complementary strands indicates that the type I intermediates containing a 6S fragment on the parental H strand were converted to completely replicated molecules, in the presence of rifampicin. These results indicate that the product synthesised in the extract by *E. coli* RNA polymerase is not necessary for completion of replication of the intermediates.

Table 1 Strand-specificity of 6S DNA in early replicative intermediates

DNA fixed on filter ³² P-DNA	³² P-DNA hybridised (c.p.m.)		³² P-DNA applied (c.p.m.)	
	H strands	L strands	Calf thymus (blank)	
Samples				
A, heated	209 (76%)	68 (24%)	2	884
B, heated	538 (67%)	278 (33%)	20	2,650
C, heated	790 (82%)	170 (18%)	10	2,907
Controls				
H strands	26 (4%)	467 (96%)	7	1,370
L strands	447 (88%)	61 (12%)	7	1,267

Early replicative intermediates labelled with α -³²P-dTMP were prepared by incubating Col E1 DNA (10 μ g ml⁻¹) and an extract of *E. coli* YS1 cells (100 μ l) in the standard reaction mixture (300 μ l) containing α -³²P-dTTP (0.5 Ci mmol⁻¹ for samples A and B; 2 Ci mmol⁻¹ for sample C), 10% glycerol and 2 mM spermidine². A freshly prepared extract was used for preparation of samples A and B. An extract which had been frozen and thawed twice during storage for a week at -20 °C was used to prepare sample C. The reaction mixture was incubated for 10 min (for sample A) or 45 min (for samples B and C) at 30 °C. DNA was extracted and early replicative intermediates (26 S) were isolated by neutral sucrose density gradient centrifugation^{2,3}. A portion of these samples was heated at 90 °C for 90 s to separate 6S fragments from the parental strands⁴. Since the amount of contaminating labelled completely replicated molecules was negligible (Fig. 1a and ref. 2), the heated samples were used to hybridise to separate strands of Col E1 DNA without further purification. The two strands of Col E1 DNA that had been treated with endonuclease *EcoRI* and denatured were isolated by CsCl density gradient centrifugation in the presence of poly(U,G)⁶. Col E1 DNA labelled with ³H-thymine (1 mCi mmol⁻¹)¹ or ³²P-H₃PO₄ (100 mCi mmol⁻¹)² was used to prepare the separate strands. ³H-labelled separate strands or denatured calf thymus DNA (0.3 μ g each) was fixed on a membrane filter⁷. Hybridisation experiments were carried out essentially as described⁷ except that three filters each of which was loaded with the H strands, L strands or calf thymus DNA were placed together in a hybridisation mixture (1 ml). After incubation for 18 h at 64 °C, the filters were washed and radioactivity was measured. The ³²P-labelled separate strands were used to test cross contamination of ³H-labelled separate strands fixed on filters. The ³²P-labelled separate strands were purer than the ³H-labelled separate strands because the concentration of ³²P-labelled strands (0.01 μ g ml⁻¹) used in the centrifugation for strand separation was much less than that of ³H-labelled strands (2 μ g ml⁻¹) used. The values in parentheses are the fractions of labelled DNA which hybridised to separate strands in the total hybridised DNA (the blank value was subtracted). Based on the results in row 4 and 5, it may be argued that the fraction of 6S H DNA in the total 6S DNA may be slightly overestimated.

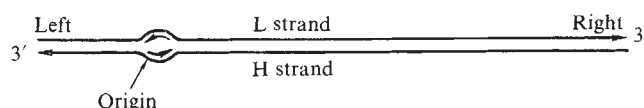


Fig. 3 Polarity of the strands of an early replicative intermediate (*EcoRI* cleaved) and the site of origin of replication.

Since most type I intermediates had a 6S L fragment and these molecules can complete replication, the site of initiation of replication of a 6S L fragment is the origin of replication of most of the molecules. The site is the 5' end of the fragment because DNA polymerase I, which extends a polydeoxyribonucleotide chain in the 5' to 3' direction⁸, is involved in the synthesis of the fragments (my manuscript in preparation). The polarity of a 6S L fragment can be deduced from that of Col E1 DNA strands, which was determined previously⁷. The 3' end of the H strand in the linear molecule created by cleavage of a closed-circular Col E1 DNA molecule by *EcoRI* is located closer to the region of early replication than the 5' end of the strand. The end of the linear molecule with the 3' end of the H strand corresponds to the left end of the molecule previously defined⁴. Therefore the 5' end of the 6S L fragment is located at the left branch point (approximately 17% of the molecular length from the *EcoRI* site^{2,4}) in the intermediate as illustrated in Fig. 3. It should be noted that unidirectional expansion of a replication loop, as was found in electron microscopic studies^{4,9,10}, does not mean that initiation of replication occurs from the fixed branch point since the method, in principle, does not reveal the site of initiation of replication and the direction of replication within the smallest replication loop observed.

Different mechanisms of initiation of synthesis of DNA strands on single-stranded phage DNAs have been reported. Initiation of synthesis of the complementary strand on M13 phage DNA is primed by *E. coli* RNA polymerase which presumably recognises a double-stranded region of the DNA¹¹. On the other hand, synthesis on ϕ X174 phage DNA and G4 phage DNA does not involve the RNA polymerase^{12,13}. In the replication of Col E1 DNA, initiation of synthesis of 6S L fragments in early replicative intermediates is completely blocked by rifampicin, while synthesis of 6S H fragments as well as further replication of the intermediates is not inhibited by rifampicin. Purified *E. coli* RNA polymerase can carry out a reaction that is equivalent to the rifampicin-sensitive reaction and is probably involved in synthesis of the primer for 6S L fragments (J. T. and T. Kakefuda, unpublished). Thus the mechanism of synthesis of the first fragment from a specific origin of replication is different from that of the fragments subsequently formed. Utilisation of the two distinct mechanisms may be advantageous by reducing the chance of aberrant initiation of replication of the molecule.

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matters arising

Empirical formulae for Student's function

THE empirical formula devised by Dawson¹ for the calculation of the theoretical values of the Student's t function at any level of probability is a useful addition for many statistical programs. I have found that the following empirical formulae provide a simple method for calculating t at various defined probability levels, P :

$$P = 0.10; t = 1.64485 + 1/(0.648n - 0.534)$$

$$P = 0.05; t = 1.95996 + 1/(0.413n - 0.423)$$

$$P = 0.01; t = 2.57582 + 1/(0.193n - 0.273)$$

where n is the number of degrees of freedom. With three or more degrees of freedom these formulae give t accurate to 0.06%, 0.1% and 0.5%, respectively. A computer program using these formulae would require only the addition of the values of t for one and two degrees of freedom. These formulae are not only more accurate than Dawson's, but are also more suitable for hand calculators.

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Cape diastrophism

IN a recent paper¹ on the Cape Fold Belt, the folded strata discussed range in age from late Ordovician² to late Permian³. Until the early Permian these strata were derived from the north^{4,5}. The ensuing palaeo-slope reversal⁶ marks the lower limit of the Cape diastrophism, and transportation of sediment from the west and south can be related to the two fold trends subsequently developed⁷. Karroo dolerites are excluded from the fold belt, and their upper age limit⁸ defines the end of the diastrophism. From the Ordovician to the early Jurassic there are very real Gondwana links: for example, the Malvinokaffric fauna⁹, the distribution of the Permo-Carboniferous glaciation¹⁰, of *Mesosaurus*¹¹, and of *Lystrosaurus*¹². The diastrophism was terminated by major faulting, and earliest marine sediments¹³ occur in the mid-Jurassic. This was the forerunner of the spreading of the South Atlantic, which commenced at this latitude about 130 Myr ago¹⁴. Before that the Falkland Plateau

lay immediately south of the Agulhas Bank¹⁵.

de Beer and his coauthors proposed¹ that an oceanic plate underthrust Gondwanaland coincident with the present southern limit of Africa, and that the Cape Fold Belt resulted from an ensuing continent-continent collision directed from the south. The data given here cannot be reconciled with this suggestion for five reasons.

First, slow shelf subsidence characterised the area for almost 200 Myr before folding. Second, there are two fold trends, one parallel to the proposed direction of collision. Third, the Agulhas Bank is considered to be floored by similar material to that present onshore¹⁶, and presents no evidence of a major suture. Fourth, the impressive similarity of the Emsian faunas of South Africa and the Falkland Islands¹⁷ suggests that the position of the latter in Devonian times was the same as in the Jurassic. Finally the concept of a plate underthrusting Gondwanaland from the south implies that Gondwanic continents such as South America and Antarctica were not in the pre-Mesozoic positions commonly ascribed to them and consequently, that the collision would involve a continent lacking Gondwana affinities. However the Cape diastrophism may have formed, a plate tectonic model is not well served by failure to consider such material evidence.

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DE BEER ET AL. REPLY—Truswell's comments¹ deal mostly with matters outside the scope of our original letter², which was intended to consider geophysical anomalies near the Cape Fold Belt and, if possible, to relate these to proposed geological models. We agree that the model suggested in the paper is simplistic and could be misinterpreted.

Although the folded strata range in age from Upper Ordovician³ to Upper Permian⁴, the main phase of folding was in the Triassic⁴. The less severe, north-south trending Cedarberg folding occurred in pre-Dwyka times⁴. The first signs of a southern land mass⁵ and pre-Dwyka folding in the east-west trending fold belt⁴ are observable in Witteberg (Lower Carboniferous) strata. This southern land mass was not simply an uplifted part of the continental craton, but an island arc type of orogenic belt, possibly related to a subduction zone south of the present coastline^{6,7}.

An important point is that the Cape Fold Belt represents only the northern edge of the more extensive Samfrau geosyncline of Gondwanaland^{8,9}. The orogenic belt and igneous rocks related to the inferred subduction zone are thus not in South Africa but lie in Patagonia and western Antarctica^{7,9,10}.

Truswell's objections to our model can be covered point by point.

First, the folding in the southern Cape occurred during Lower Carboniferous times, which means that shelf subsidence lasted only about 100 Myr before the start of any orogenesis related to subduction. Second, the degree and type of deformation accompanying continental collision (or continent-island arc collision) depend on the shapes of the continents and the morphology of the trench system, or systems, involved (see, for example, ref. 11). A fold system with only two fold trends of different ages will require a relatively simple model. The relevant geological and geophysical evidence from South America and Antarctica must be included in such a model. Third, this indicates that the

suture will be further south. Fourth, geophysical evidence strongly suggests an Upper Triassic–Lower Jurassic Gondwana model in which the Falkland Plateau as part of the South American plate borders the African plate^{12–14}. This model is consistent with Truswell's considerations and with the hypothesis that the Falkland islands were to the north of the inferred subduction zone. The position of the subduction zone with respect to Patagonia is unclear. If it was situated to the east of Patagonia, the suture could lie in the basement structures in the continental edge and in the Malvinas Basin¹⁵. The suture could then cross the continent in the Upper Permian Patagonian Massif⁸ or the Middle Triassic Deseado Massif¹⁶. Geological evidence, however, strongly supports the existence of a subduction zone that bordered the western rim of Patagonia from Devonian to Triassic times^{7,17}.

Fifth, from the Gondwana model it is clear that parts of Patagonia and the Antarctic Peninsula formed the southern land mass that approached southern Africa during Palaeozoic times in a process peripheral to the main Gondwana land mass. Even this accreted land mass should show affinities with Gondwanaland from at least Lower Carboniferous times.

This first order extension of our very elementary model inferring a continental collision origin for the Cape Fold Belt shows that the constraints proposed by Truswell can indeed be reconciled with a continent–continent (or at least a continent–island arc) collision south of Africa.

Our explanation of the origin of the Beattie magnetic anomalies is more contentious and should perhaps be related to processes causing the accretion of the present-day basement in the Karroo and further south rather than to processes causing the formation of the fold belt.

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Chocolate, β -phenethylamine and migraine re-examined

ON the basis of an industrial analytical report indicating that chocolate might contain as much as 3 mg of β -phenethylamine (PEA) per 2-ounce bar, Sandler *et al.*¹ administered 3 mg of this amine to each of 36 chocolate-sensitive migraine-prone subjects and found that 18 suffered attacks within 12 h, as opposed to six attacks with placebo.

zene-ethyl acetate (9:1). A portion of the eluted radioactive zone was counted and a smaller portion was injected on to a column containing 3% Poly A-103 on Gas Chrom Q (F and M Model 400, fitted with Hewlett-Packard ⁶³Ni electron capture detector). With the use of external standards prepared from crystalline N-heptafluorobutyl-PEA and an estimate of losses provided by recovered ¹⁴C, a simple calculation provided the native PEA level.

As Table 1 shows, the highest level, 780 μ g per 2 ounce, was found in the unsweetened variety. Semi-sweet forms contained intermediate amounts and milk chocolate was lowest in PEA. The decline may be related to the amount of time the raw chocolate is subjected to conching, a mellowing process during manufacture which includes rolling and heating. PEA, a relatively volatile amine, could escape during this step. The modest rise in PEA after relatively severe hydrolysis indicates that presumed conjugates do not contribute substantially to the total potential PEA pool.

Table 1 PEA levels in various brands of chocolate

Brand	Country	Type*	μ g PEA-HCl per 2 ounce		Recovery of PEA-HCl added to 1 g chocolate		
			First analysis	Second analysis	Added (μ g)	Native level (μ g)	Total recovered (μ g)
A	England	Milk	38	41	—	—	—
		Mild sweet	285	315	1.0	5.0	6.2
B	USA	Milk	78	85	50.0	1.4	57.0
		Mildly sweet	330	—	—	—	—
C	USA	Milk	21	—	—	—	—
		Semi-sweet	360	390	1.0	6.4	8.2
D	USA	Unsweetened	780	978†	20.0	13.8	35.7
E	Switzerland	Milk	86	—	—	—	—
		Bittersweet	260	—	—	—	—
F	Switzerland	Milk	103	—	—	—	—
		Bittersweet	340	—	—	—	—

*Manufacturer's description.

†Sample subjected to 100 °C hydrolysis in 6 M HCl for 0.5 h.

Having previously developed a sensitive procedure for the analysis of PEA in urine², we applied it with minor modifications to the analysis of one US brand of milk chocolate. We found only 21 μ g per 2-ounce bar, or about $1/150$ of that quoted above. Various brands and types of chocolate were then examined.

In the method, 1-¹⁴CPEA, 8.86 mCi mmol⁻¹, was used as an internal standard. About 10⁵ d.p.m. were added to 1–2 g chocolate melted in 5 ml water. With some samples, 1–50 μ g of unlabelled PEA was also added in tests to demonstrate recovery. Briefly (see ref. 2 for details), bases were extracted with chloroform. After concentration, the residue was applied to a silica gel plate and developed in ethylacetate–methanol–diethylamine (9:1:1). The labelled area, eluted and derivatised with heptafluorobutyric anhydride, was chromatographed in ben-

These data suggest that chocolate-induced migrainous attacks are either not related to PEA-induced headaches, or that patients suffering from migraine are sensitive to very low levels of PEA.

This work was supported by a Research Scientist Award to A.J.F. and a grant from the National Institute of Mental Health.

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reviews

THE first volume of this textbook, dealing with the principles of the subject, appeared in 1962 and reached a second edition in 1968. Good of its kind, it necessarily follows fairly conventional lines. The second volume aims at global history in a way that has never previously been attempted. It was about three-quarters complete when H. H. Read died in 1970, in his 80th year; Professor Janet Watson is to be congratulated on bringing the eagerly-awaited work to fruition.

The method adopted is to integrate the broad record of stratigraphy of rocks formed at or near the Earth's surface with structural, magmatic and metamorphic events which occurred at depth within the crust. The evidence comes fundamentally from geological mapping at the surface, with data on the third dimensions derived from valleys, mines, boreholes and the indirect probing of geophysics. The process has been carried to a very variable degree in different parts of the world, but there is no doubt that the time is ripe for synthesis on the scale attempted here. Moreover, the classical method of time-assessment from fossils has now been carried far back into the Cryptozoic, and the newer method of radiometric dating gives a valuable control over Cryptozoic and Phanerozoic alike, once its limitations have been appreciated. The authors point out, however, that they have had to place severe limitations upon the amount of stratigraphic (and, therefore, palaeontological) detail given. The framework is, in fact, not stratigraphy but tectonics and this follows very suitably from the last chapter of Volume 1 ("A pattern of earth history") which drew a sharp distinction between stable regions of the crust and belts of mobility. The history of the

New geological framework

Introduction to Geology. Vol. 2: Earth History. By H. H. Read and Janet Watson. Part 1: *Early Stages of Earth History*. Pp. xii+221. £5.95 board; £2.95 paper. Part 2: *Later Stages of Earth History*. Pp. xii+371. £6.95 board; £3.95 paper. (Macmillan: London and Basingstoke; distributed in the USA by Halsted Press; May 1975.)

mobile belts is adopted as the framework for Volume 2, though the cratons of Precambrian time, the interior lowlands, epicontinental basins and forelands are by no means forgotten.

Volume 2 is issued in two parts in order to make possible the publication of the paperback edition. Part I deals with the first 2.9×10^9 years, covering Precambrian events up to the later Proterozoic. As far as I know, this is the first time that a global synthesis of the Precambrian, involving all the continents including Antarctica has been attempted. For each continent, an outline of the tectonic pattern is first given; then the stages by which the rocks older than 900 Myr have been formed is set forth. A concept of a series of cycles emerges; the ending of a cycle brings stability to tracts that were formerly mobile, contributing to the growth of cratons; but the initiation of a new cycle may affect tracts that were formerly stable.

Many interesting problems for future solution come directly from this synthesis.

Though it seems unlikely that a stratigraphic classification like that adopted throughout the world for the Phanerozoic will ever become feasible for the ancient rocks, some correlations certainly begin to emerge, as Figure 10.1 shows in the book. Indeed Part I will make good reading for those involved in the Precambrian priority area of the International Geological Correlation Programme of UNESCO/IUGS.

Part II commences with a chapter on new themes in Earth history which provides a convenient way of dealing with continental drift and palaeoclimatic change. The method used in Part I is then readopted and the framework again established on mobile belts, but belts which will be much more familiar to most readers than those described in Part I: the Caledonian, Hercynian-Uralide, Alpine-Himalayan and Cordilleran. The interior platforms of little distorted cover-rocks resting on Precambrian or later basement receive due attention, and the break up of the great ancient southern continent of Gondwanaland is described. This leads on to the current concepts of global tectonics, the opening of the Atlantic and Indian oceans, and the outward circumferential push around the Pacific.

Even if the authors have had to scale down the over abundant geological detail, they have succeeded in following those aspects of geological process that can be traced throughout the full span of Earth history, at the same time pointing a finger at a few, like banded ironstone formation or the detrital transport of uraninite, that long ago ceased for ever. Surprisingly in this historical essay, the useful rocks and minerals are not forgotten, but appear in their due genetic positions.

Kingsley Dunham

TRANSPORT of peptides across biological membranes, first discovered in microorganisms, is now recognised to be of major significance in the intestinal absorption of amino-N, yet little is known of other roles of peptide transport.

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Peptide transport

Peptide Transport in Protein Nutrition. (North-Holland Research Monographs, Frontiers of Biology, Volume 37.) Edited by D. M. Matthews and J. W. Payne. Pp. xxii+503. (North-Holland: Amsterdam and Oxford; American Elsevier: New York, 1975.) Dfl. 130; \$54.25.

nificantly to the flavour of foodstuffs. Some taste extremely sweet, others containing acidic and neutral amino

acids taste sour but (see page 55) Mrs Beeton's recipe for tasty beef tea produces a satisfactory mixture.

The Editors and their eight colleagues have succeeded in delving into diverse disciplines to produce a generally well-balanced account of knowledge of peptides, with the emphasis on nutrition.

This is an expensive book (10 pages = \$1.00), but an essential acquisition for libraries. It will be read with profit by biochemists, clinicians, nutritional scientists and food technologists.

Dennis Parsons

Guide to the Marine Stations of the North Atlantic and European Waters. Part I. Northern Europe and the East Atlantic Coast. Compiled by J. E. Webb. Pp. 263+34 figs. (The Royal Society; London, 1974.) £2.50 (UK); £2.60 (overseas).

THIS guide, compiled by Professor J. E. Webb, is the first of a new series of handbooks which will be extended in further parts to cover marine stations of the Mediterranean, the Atlantic seaboard of USA and Canada, and the Caribbean and Gulf of Mexico.

Part I is an invaluable source of information on the facilities offered at 33 European marine stations, including 12 within the United Kingdom and Eire. The information is concise and practical. Short descriptions are given of the main types of shores to be found in the neighbourhood of the stations; of the common animals that can be obtained without difficulty; at the time of year when they occur; of the ships, boats and vehicles that are available for field surveys; and of the types of gear at hand for the collection of specimens and data.

The number and nature of the services provided in the research rooms and laboratories of the station are listed. The availability of special apparatus, such as is often required by physiologists, is briefly but usefully noted; and an intending visitor may be guided on the nature of the apparatus, equipment and materials which a station may be able to provide. Notes on the voltage, d.c./a.c. frequency, and phase of the electricity supply at the various stations are especially useful. Excellent outline maps showing the locations, the nature of the shorelines and the approaches to stations, illustrate the guide.

The guide, as noted in the preface, is not intended to be comprehensive. Some additional information would perhaps have been useful, such as the languages commonly spoken, the technical assistance that a visitor might expect to receive and fuller notes on the characteristics of the plankton and algae. The guide is, however, in a form which lends itself to ready revision as the need arises, and in its present form it is up-to-date and informative. **J. E. Smith**

Comprehensive Virology. Edited by Heinz Fraenkel-Conrat and Robert E. Wagner. Vol. 2: Reproduction—Small and Intermediate RNA Viruses. Pp. xiv+340. \$33.60. Vol. 3: Reproduction—DNA Animal Viruses. Pp. xiv+488. \$38.50. Vol. 4: Reproduction—Large RNA Viruses. Pp. xii+347. \$34.90. (Plenum: New York and London, 1974-75.)

VOLUME I of *Comprehensive Virology* was essentially a catalogue, containing a very limited amount of descriptive material. The next four volumes, of which volumes 2, 3 and 4 are now published, are entirely different from Volume 1, and are all concerned with the ways in which viruses reproduce themselves. These are major reviews, and although one or two contributions show signs of being variations upon earlier texts by the same author(s), much of the work is new and up to date. The sections in Volume 2 by E. R. Pfefferkorn and D. Shapiro on togaviruses, and in Volume 3 by B. Roizman and D. Furlong on herpesviruses, are particularly fresh and original. Later volumes will deal with structure and assembly, and with regulation and genetics. This approach means that a virologist with an interest in one particular virus family is obliged to buy three volumes in order to cover all aspects of his particular virus. By so doing, however, he is likely to read more widely than if he confined himself to single-virus books on 'poxviruses' or 'herpesviruses', and will thus benefit himself as well as the publishers. **J. S. Porterfield**

Books brief

Introduction to Geochemistry. (Geophysics and Astrophysics Monographs, vol. 10.) By Claude-Jean Allègre and Gil Michard. Pp. viii+142. (Reidel: Dordrecht and Boston, Massachusetts, 1974.) n.p.

Handbook of Geochemistry, vol. 2, part 4. Edited by K. H. Wedepohl. Pp. vi+898. (Springer: Berlin and New York, 1974.) DM.298; \$122.20.

WERE he to judge from the title the tyro might expect *Introduction to Geochemistry* to provide a comprehensive appraisal of geochemistry at a reasonably elementary level. Instead, he will find a good introduction to certain important geochemical processes. The book confines itself essentially to element fractionation: the fractionation of major and trace elements through magmatic processes and in the hydrosphere; the fractionation of light isotopes, with particular reference to $^{18}\text{O}/^{16}\text{O}$ and $^{32}\text{S}/^{34}\text{S}$ isotopic compositions; and of radioactive isotopes. Natural processes in which chemical equilibrium is not attained are also considered.

The book is a translation, "done without amendments, editings or alterations", and, consequently, it appears as a translation, although the

sentence, 'A test of the validity of these equations can be gotten here in a simple way' conjures up some thoughts about the original French. The authors' "rule" to omit many text citations from the bibliographies is irritating.

The *Handbook of Geochemistry*, the latest part of a continuing project, upholds its high standard of content and production, and meets its publication date. The authors, editors and publishers are all to be congratulated on maintaining the impetus of the project. Chapters covering a further 24 elements (including carbon, nitrogen, oxygen, silicon, magnesium and iron) are now supplied; little is missing from another 21 chapters; and with more than three quarters of the elements completed only relatively small gaps remain. Inevitably, with publication over a six-year period, costs have escalated, but at least fairly uniformly. The price is now 14.7 cents for each page, compared with a price of 5.5 cents in 1969. The *Handbook of Geochemistry* is invaluable to earth scientists, but regrettably, it is a library purchase, and these days even some libraries may resist temptation. **Duncan Murchison**

Fast Reactions. (Oxford Chemistry Series.) By J. N. Bradley. Oxford Chemistry Series (Number 23). Pp. x+121. (Clarendon: Oxford; Oxford University Press: London, June 1975.) £3.25.

PROFESSOR Bradley has written a very concise description of fast reaction kinetic studies, emphasising throughout his book the experimental rather than the chemical aspects of the subject. The descriptions of the individual techniques are not very detailed, and the author's own interest in gaseous, rather than solution work, is very apparent. A preferable presentation would have been to split the subject into two parts of this series, one referring to gaseous the other to solution kinetics. In any case the value of the book would be greatly enhanced by the provision of many more up-to-date chemical examples, particularly for the solution kinetic techniques. The standard of production of the book does not compare favourably with others in the series: the print was blurred on many of the pages of the reviewer's copy, and the line lengths had not been justified. A number of diagrams need better description or more labelling, and there are a few text errors. The reviewer will continue to use Caldin's *Fast Reactions in Solution* (1964) and Hague's *Fast Reactions* (1971) as preferred recommended texts.

John Maher

Bonded states

Metal-to-Metal Bonded States of the Main Group Elements. By M. J. Taylor. Pp. 211. (Academic: New York and London, May 1975.) \$15.25; £5.80.

COMPOUNDS in which ligands are linked to a cluster of atoms, instead of to a single atom, have opened up much novel chemistry in recent years. Much of this has been in transition metal chemistry, but cluster compounds spread right across the periodic table, and interest in them stems from their novel compositions, the apparently low valence states of the elements, their stereochemistry and their valence-theoretical description.

Dr Taylor has explicitly restricted himself to compounds of the main group elements. In addition, he limits consideration to compounds of elements that, in the elementary state are regarded as metals. There is no real justification for doing so, and the arbitrary restriction has some illogical consequences. Thus, he has to discuss the cationic clusters formed by selenium and tellurium without proper reference to their exact analogues in sulphur chemistry, and he makes no mention at all of the anionic clusters formed by silicon and germanium. But equally mistakenly in my opinion he broadens his survey to include all compounds in which two 'metal' atoms are linked. The real interest of metal-metal bonding surely resides in the consequence of electron delocalisation within a cluster or chain of identical (or at least essentially interchangeable) atoms. The pro-

perties of, for example, the $(\text{CH}_3)_3\text{Sn}$ group, like those of the $(\text{CH}_3)_3\text{Si}$ or even the $(\text{CH}_3)_3\text{C}$ group, are those of a straightforward σ -bonding group; there is nothing special about such compounds as $(\text{CH}_3)_3\text{SnCo}(\text{Co})_4$, or $(\text{CH}_3)_6\text{Sn}_2$, to justify special attention to the 'metal-metal' bond. The amount of space devoted to compounds of this kind dilutes what should be the main interest of the book.

What the book does provide is much information on the formation of dimer and cluster species, in solutions and melts, and structural information based on vibrational spectroscopy and crystal structure. It has a thorough bibliography, heavily biased towards work of the last 15 years, including references up to 1973. This review content is its main value. For student reading, it does not bring a unifying outlook to a diverse field; for example, there is repeated terminological confusion between the oxidation states, which may often be experimentally determined, and the valence states of elements which in cluster compounds are certainly different from the oxidation states and which may be debatable. There are many typographical errors, but few will lead to confusion. Readers will note that, on the dust jacket, Academic Press have now decreed that selenium and tellurium shall be Group Vb elements.

The author states that the book is an expanded treatment of a review. One must query whether that expansion was really justified. A good review does not necessarily produce an equally good monograph.

J. S. Anderson

Literature on water

Water and Aqueous Solutions: Introduction to a Molecular Theory. By Arieh Ben-Naim. Pp. xvi+474. (Plenum: New York and London, 1974.) \$34.90.

THE basic thesis of this book is that some sort of mixture model provides a fundamentally correct physical description of the thermodynamic properties of water and aqueous solutions and that mixture models can be made respectable or rigorous by embedding them in or deriving them from the fundamental statistical mechanical theory of fluids. The author is not particularly concerned with establishing the validity of any particular mixture model. Rather, he is concerned with the features common to all such models and with giving precise statistical mechanical definitions of the concepts used in discussing them.

The first half of the book discusses the statistical mechanics of fluids, molecular distribution functions, and the theory of solutions. The last half is concerned with liquid water, water with one simple (non-hydrogen-bonding) solute, and

water with two simple solutes. The emphasis in this second half is almost entirely on thermodynamic properties, rather than on time dependent or spectroscopic measurements. A major limitation of the book (which is a reflection of when its writing was completed) is that it contains only a brief discussion of the earliest computer simulation studies of realistic models of water. Such studies should provide the most convincing evidence for or against the usefulness of mixture models.

Many of the features of the book are of interest and of value even to the reader who does not share the author's preference for mixture models; for example, the careful discussion of those aspects of solution theory which are needed to understand experiments on aqueous solutions, the precise discussion of standard thermodynamic quantities of transfer, and a statistical mechanical definition of the concept of hydrophobic interaction. Though the book does not answer many questions it does formulate many about water and aqueous solutions in a precise way. It is a useful companion volume for the large recent review literature on water.

Hans C. Andersen

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H. C. Hottel and **H. Tabor** have jointly been awarded the 1975 **Royal Society Esso Award for the Conservation of Energy** for their work on solar energy collection.

Appointments

Sir Andrew Huxley is to be the 1976–1977 president of the British Association for the Advancement of Science.

J. S. Anderson has been appointed Honorary Professorial Fellow and **J. L. Hutchison** Research Associate to the Solid State Chemistry Group at the Edward Davies Chemical Laboratories, University College of Wales. The Science Research Council has awarded a grant to support their research.

International meetings

November 3–5, **Refuse-to-energy**, Montreux, Switzerland (Professor N. Y. Kirov, University of New South Wales, Department of Fuel Technology, PO Box 1, Kensington, NSW 2033, Australia).

November 3–6, **Advances in chromatography**, Munich, Germany (Professor A. Zlatkis, Chemistry Department, University of Houston, Texas 77004, US).

November 3–6, **High energy particle interactions**, Smolenice, Czechoslovakia (Dalibor Krupa, Institute of Physics, Slovak Academy of Sciences, Dubravska Cesta, CS-89930, Bratislava).

November 3–7, **Biological effects of low level radiation pertinent to protection of man and his environment**, Chicago, US (International Atomic Energy Agency, PO Box 590, Kartner Ring 11, A-1011, Vienna, Austria).

November 3–7, **Biomedical transducers**, Paris (Secretariat, International Conferences, c/o F.N.I.E., 16, rue de Presles, F-75740 Paris Cedex 15).

November 3–7, **CIOS congress**, Caracas, Venezuela (International Council for Scientific Management, 1 rue de Varembe, BP 20, CH-1211, Geneva, Switzerland).

November 4–8, **Molecular basis of circadian rhythms**, Berlin (Dr S. Bernhard, Dahlem Konferenzen, 1 Berlin 33, Delbruckstrasse 4c, Germany).

November 9–15, **American water sources**, New Orleans (AWRA, St Anthony Falls Hydraulic Laboratory, University of Minnesota, Mississippi River, Third Avenue, Minneapolis, Minn. 55414, US).

November 10–11, **Energy saving in chemical processes**, Milan (FAST, Piazzale R. Morandi 2, 120 Milan, Italy).

November 10–13, **Sensing environmental pollutants**, Las Vegas (B. H. Mannheimer, Department of Housing and Urban Development, Washington D.C. 20410, US).

November 16–19, **Association of earth sciences, editors annual meeting**, Hershey, Pa. (J. P. Wilshusen, Pennsylvania Geological Survey, Department of Environmental Resources, Harrisburg, Pa. 17120, US).

November 16–20, **Laboratory animal science**, Boston, Massachusetts (Mr J. J. Garvey, AALAS, 2317 W. Jefferson Street, Suite 208, Joliet, IL. 60435, US).

November 16–26, **Human environment**, Kyoto, Japan (Science Council of Japan, 22–34 Roppongi, 7 Chome, Minato-Ku, Tokyo).

November 17–20, **Insecticides and fungicides**, Brighton (Mr W. F. P. Bishop, Conference Secretary, 87 London Road, Croydon CR0 2RF, UK).

November 17–21, **Transuranium nuclides in the environment**, San Francisco (International Atomic Agency, PO Box 590, Kartner Ring 11, A-1011, Vienna, Austria).

Reports and publications

Other countries

World Health Organization. Technical Report Series. No. 564: Organization of Mental Health Services in Developing Countries—Sixteenth Report of the WHO Expert Committee on Mental Health. Pp. 41. Sw. fr. 6. No. 570: Viral Hepatitis—Report of a WHO Meeting. Pp. 51. Sw. fr. 7. Guide to the Laboratory Diagnosis of Trachoma. Prepared by the participants in a WHO Symposium. Pp. 38. Sw. fr. 12. (Geneva: WHO; London: HMSO, 1975.) [177]

World Health Organization. Understanding Research in Nursing. By Shirley Chater. (WHO Offset Publication No. 14.) Pp. iii + 36. (Geneva: World Health Organization; London: HMSO, 1975.) Sw. fr. 6. [187]

An Economic Analysis of the Co-operative Medical Services in the People's Republic of China. By Teh-wei Hu. (A publication of the John E. Fogarty International Center for Advanced Study in the Health Sciences. US Dept. of Health, Education and Welfare, Public Health Service, National Institutes of Health.) Pp. 41. (Washington, DC: Government Printing Office, 1975.) 75 cents. [187]

Canada: Department of Energy, Mines and Resources. Geological Survey of Canada. Bulletin 235: Contributions to Canadian Paleontology (three papers). By A. J. Boucot, J. G. Johnson, Rolf Ludvigsen and D. G. Perry. Pp. 106 (19 plates). \$6. Bulletin 248: Conodonts of the Hull Formation, Ottawa Group (Middle Ordovician), Ontario and Quebec. By T. T. Uyeno. Pp. 31 (3 plates). \$2.50. Paper 74-21: Reconnaissance Geology of a Part of the Precambrian Shield, Northern Quebec and Northwest Territories. By F. C. Taylor. Pp. 10. \$2. Paper 74-38: Illustrations of Canadian Fossils. Cretaceous Foraminifera from Saskatchewan and Manitoba. By B. R. North and W. G. E. Caldwell. Pp. iii + 35 (12 plates). \$4.20. Etude 74-42: Une Méthode d'Utilisation Quantitative de la Sonde Neutron-Neutron (Porosité) pour l'Étude des Dépôts Meubles. Par Jacques Locat. Pp. 43. \$3. Paper 74-51: Seismic Structure of the Continental Margins and Ocean Basins of Southeastern Canada. By R. Jackson, C. E. Keen and M. J. Keen. Pp. 13. Paper 74-59: Magnetic Properties of Pyrrhotite and Their Use in Applied Geology and Geophysics. By E. J. Schwarz. Pp. 24. \$3. Paper 74-62: Cavendish Township Geophysical Test Range—1973 Diamond Drilling. By D. A. Williams, W. J. Scott and A. V. Dyck. Pp. 14. \$3. Paper 75-8: Catalogue of X-ray Diffraction Patterns and Specimen Mounts on File at the Geological Survey of Canada. By M. Bonardi and R. J. Traill. Pp. 51. \$3.60. (Ottawa: Information Canada, 1974 and 1975.) [187]

Bibliography of Science Material on History of Science and Technology in Medieval India—an Introduction. By Professor A. Rahmann. Pp. 9. (New Delhi: National Commission for the Compilation of History of Sciences in India, Indian National Science Academy, 1975.) [217]

Unesco. Annotated Bibliography of Textbooks and Reference Materials in Marine Sciences. Provisional edition. (Intergovernmental Oceanographic Commission Technical Series.) Pp. 109. (Paris: Unesco, 1975.) [217]

Smithsonian Contributions to the Earth Sciences. No. 15: Sands in the Alboran Sea—a Model of Input in a Deep Marine Basin. By Daniel Jean Stanley, Gilbert Kelling, Juan-Antonio Vera and Harrison Sheng. Pp. iii + 51. (Washington, DC: Smithsonian Institution Press, 1975. For sale by US Government Printing Office.) \$1.20. [227]

The Institute of Cancer Research, Philadelphia. Nineteenth Scientific Report, September 1973–September 1974. Pp. 267. (Philadelphia: The Institute for Cancer Research, 1975.) [237]

Unesco. Intergovernmental Oceanographic Commission Technical Series. No. 13: The International Decade of Ocean Exploration (IDOE) 1971–1980. Pp. 87. (Paris: Unesco, 1975.) [237]

CERN—European Organization for Nuclear Research. CERN 75-8: Problems of Classical Dynamical Systems. By W. Thirring. Pp. 28. (Geneva: CERN, 1975.) [237]

Smithsonian Year 1974: Annual Report of the Smithsonian Institution for the year ended June 30, 1974. Pp. viii + 500. (Washington, DC: Smithsonian Institution Press, 1974. For sale by US Government Printing Office.) \$6.65. [247]

The International Federation of Institutes for Advanced Study, Stockholm—Descriptive Brochure. (Stockholm: IFIAS, 1975.) [247]

Respiratory Research in the People's Republic of China. By Frederick F. Kao. (A publication of the John E. Fogarty International Center for Advanced Study in the Health Sciences. US Department of Health, Education and Welfare, Public Health Service, National Institutes of Health. Pp. xii + 141. (Washington, DC: US Government Printing Office, 1975.) \$1.75 [247]